BIOLOGICAL INDICATORS OF FOREST DIEBACK.

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN COLLABORATION WITH THE FORESTRY COMMISSION.

THE ENVIRONMENTAL RESEARCH UNIT
THE DEPARTMENT OF SCIENCE AND CHEMICAL ENGINEERING
THE UNIVERSITY OF GLAMORGAN
DECEMBER 1992.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. K. W. Burton, Dr. E. Morgan and Dr M. Coutts for their help and encouragement during the course of this project. I would like also, to thank Mr. N. Danby and the others who have helped me at the Brecon Research Station.

Thanks are due to Dr Ryszard Babecki and Miss Yvette Wright who have helped in translation of some of the papers referenced in this Thesis. I must also thank Dr. A. J. Berry for his valuable assistance with the SFC work. A special thankyou is due to Robert Henly who wrote a program for me and also gave advice on how to use Word 2 and Excel 4.

I must also thank Mr. D. Clark for acting as chauffeur on the cold jaunts around the Afan 1 Forest. I am grateful for the help that Dr. J. Winter, Kevin, Phil and the rest of the technical staff of the department have provided. I must also thank Alvine and Mike for the loan of the bench centrifuge when I needed it!

Declaration

This thesis has not been nor is being currently submitted for the award of any other degree or similar qualification.

D. P. Evans

For my parents

ABSTRACT

This study involved the determinations of the foliar levels of chlorophyll a and b and metal levels in the year 1, year 2 and year 3 needles of Sitka spruce. A survey was made of nineteen sites in the Afan 1 forest in South Wales. The results obtained indicates that there are two distinct types of site, namely good growth sites and poor growth sites. The poor growth sites were found to be at higher elevations.

Further analyses of the results show that there is possibly an increased loss of nutrients from the trees at the poor growth sites. This may be due to increased pollution that may be present at the higher elevations.

In 1987 a survey was made of the one year old epicormic tissues that were compared with the normal tissues at the 19 sites of the Afan 1 Forest There were no statistically significant differences in the levels of the chlorophylls or in any of the metals that were looked at.

At the Afan 1 Forest there are observed pairs of trees that are growing quite close together, but they exhibit quite a marked difference in growth. One of the pair shows vigorous healthy growth, whilst the other is stunted and shows symptoms of decline. One such pair is found at Site 15. They are of the same age and from the same seed stock. It was found that over an eight month period, the stronger growing of the pair had significantly higher levels of chlorophyll a and b, potassium, calcium and magnesium. This may indicate increased leaching and foliar damage from the poorer growing of the pair.

Three more pairs of trees together with their grafts were examined from sites' 1,5 and 6, for their levels of peroxidase enzymes. Peroxidase has been used to determine possible genetic differences in cultivars. The work yielded very little useful information.

The SCOR-UNESCO equations have been widely used as a means of determining chlorophyll levels from plant extracts. A multivariate calibration method was used in conjunction with HPLC to determine the accuracy of these equations for determining the levels of chlorophyll and b. The results show revealed that the equations appear to be underestimating the chlorophyll a levels. Leading on from the HPLC work, a novel method for the separating a mixture of chlorophyll a and b was developed using packed column SFC. The method appears to be quite promising with its better resolution and faster analysis times as compared to HPLC methods.

Sitka spruce seedlings were grown in COIR (coconut husk) and were used in and were used in a central composite design to determine the effects of cadmium and copper, both singly and in combination would have upon their growth. Response surface methodology was used to determine the effects and it was found from the experiment that high levels of cadmium appeared to have an effect upon the elongation of the needles of the new shoots.

Glossary of Terms

AAS	Atomic Absorption Spectrophotometry
a.u.f.s	Absorbance Units Full Scale Deflection
CAI	Current Annual Increment
CEGB	Central Electricity Generating Board
Chl	Chlorophyll
corr.	Correlation
df	Degrees of Freedom
Diff.	Difference
GYC	General Yield Class
Ht	Height
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatograph
MAI	Mean Annual Increment
M.P.	Modelling Power
Pa	Pascals
PAGE	Polyacrylomide Gel Electrophoresis
PC	Principal Component
P_c	Critical Pressure
PCA	Principal Component Analysis
PGYC	Present General Yield Class
PLS	Partial Least Squares Principal Component Regression
Prob.	Probability
psi	Pounds Per Square Inch
S(St)	Site
S^2	Variance
SA	Specific Activity
sd	Standard Deviation
se	Standard error
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SIMCA	Soft Independent Modelling of Class Analogy
T_c	Critical Temperature
TLC	Thin Layer Chromatography
UV	Ultra Violet
Vis	Visible
Yr(yr)	Year

INDEX

LIST OF TABLES

LIST OF FIGURES.

CHAPTER 1

1. INTRODUCTION.

During the last decade or so, there has been growing concern at the decline of forest vitality in many regions of Europe and North America. The problem was identified as a 'new type' of forest decline in W.Germany, different from the well-documented declines (particularly in fir) of earlier decades [Ashmore et al (1985), Cape et al (1988)]. This loss of vitality has been observed on rich/poor, acid/basic, wet/dry soils. This 'new type' of forest decline has many unique features, such as appearing suddenly and showing symptoms on different tree species coniferous as well as deciduous, and occurring over large geographical areas. It also involved widespread abnormal growth responses.

In Germany, specific groups of damage symptoms have been characterised at specific areas. These include the yellowing of needles of spruce trees growing at both low and high altitudes in the Alps, distinct dieback and thinning of the trees at medium and high altitudes such as in the Harz Mountains, and the reddening of needles of older trees growing in the southern parts of Germany [Guderian et al (1985)]. This realisation led to the setting up of a nation-wide survey in W. Germany, which showed a progression from 1982 to the point where half the forests in the country were showing signs of this 'new type' of decline by 1985, with no widespread evidence of recovery [Rose (1985)]. The symptoms of the decline appeared randomly at first, usually in trees that were over sixty years old and at elevations of 600 m or so, near both sides of the Czechoslovakian border and in the Black Forest [Treshow and Anderson (1989)].

Many of the symptoms of this 'new type' of decline have been observed at the Afan 1 Forest (the area used for this study). The Afan 1 Forest is situated in the area of Glyncorrwg that is just west of the Rhondda Valley in South Wales (Fig. 1.1 and Fig. 1.2 is a map of the sites at the Afan 1 Forest). One of these symptoms is 'bent top', where the apical shoot loses its negative geotropism and this is not related to wind direction [Coutts (Pers. Comm.) (1986)]. There is also evident on many of the trees a chlorotic mottling. Such mottling has been attributed to ozone damage [Skeffington and Roberts (1985)].

Older needle chlorosis and needle loss have been observed in many of the stands. This maybe due to various factors such as nutrient deficiencies [Binns et al (1980)], or possible 'heavy metal' contamination [Morgan (1983), Coutts (Pers. Comm.) (1986)] The characteristic blue-green colour of the spruce is not present in many of these trees and this may be due to the loss of the waxy cuticle that helps to prevent excess water loss [Pers. Comm. (1986)]. Acid rain has been implicated in destroying the epicuticular wax [Percey and Baker (1990)]. The epicuticular wax is thought to play an important role in a tree's winter hardiness and drought resistance [Percey and Baker (1990)]. Changes in the waxy cuticle have also been reported in areas where there is air pollution [Percey and Riding (1978), Sauter and Voss (1986)]. Ozone is another possible cause of damage to the waxy cuticle [Percey et al (1990)].

Another symptom of forest decline observed at the Afan 1 Forest has been an increase in the number of epicormic shoots [Coutts (Pers. Comm.) (1986)]. These epicormic shoots are produced when apical dominance is lost i.e. in response to defoliation due to pollution or mechanical damage [Rose (1985)]. This little known phenomenon has been called fear or 'Angst' twigs because of the characteristic shape taken by the trees that develop them.

1.1 What is Meant by Forest Decline?

First there occurs chlorosis of older leaves, which in many instances leads to premature defoliation. The younger needles of conifers progressively become more yellowed (an indication of chlorosis) and drop, and finally the shoots die back to produce a thinning canopy. Trees become increasingly weakened with a decrease in the rate of growth, and often over a number of years the tree dies, generally starting at the top. Mature trees are most often affected. this is not a specific picture though, since the symptoms vary in different regions of Europe and North America [Cape et al (1988), Chevone and Linzon (1988), Treshow and Anderson (1989)].

A number of terms are used in conjunction with decline. One such term is dieback, which involves the death of outer twigs and limbs [Treshow and Anderson (1989), Cape et al (1988)]. It can be a symptom of decline but it is not the whole picture. Disease is a broader term and involves any departure from normal health, it is usually assumed that some biotic factor is responsible. Injury is another term used, and it refers to a local damage such as those which may be a result of insects or mechanical injury e.g. frosts or wind [Nihlgard (1985), Treshow and Anderson (1989)].

1.2 What are the Causes of Forest Decline?

There are a number of major ideas trying to answer this question. Hypotheses have been put forward relating this decline to various stresses. These include acid rain, ozone, acid mists and air pollution [Mudd (1975), Nihlgard (1985)] It is most probable that the decline is a combination of these various factors. Climatic factors such as sudden drops in temperature, warm periods in winter, drought, ice storms or hurricanes bringing salt from the sea, all can impair plant health [Treshow (1970)]. Atmospheric ammonia has been suggested as a possible cause. The ammonia (excess nitrogen) can be deposited far from its point of source. Modern farming methods may be to blame for some of the excess

nitrogen being deposited on forests [Nihlgard (1985)]. Excess nitrogen being taken into the plant in the form of ammonium ion, nitrate or nitrite can cause the plant to use up its carbohydrate reserves [Nihlgard (1985)]. The excess of nitrogen can result in rapid growth and elongation of cells and the plant may come to a point where it may not be able to support its own weight [Nihlgard (1985)].The increase of ammonium ion in the soil also appears to inhibit the growth of symbiotic fungi which in turn leads to a reduced uptake of potassium and magnesium by the plant [Treshow and Anderson (1989)].

The widespread occurrence of the decline may implicate some global or regional weather phenomenon such as drought that may have served as a 'triggering' mechanism that predisposed trees to secondary stresses [McLaughlin (1985)]. Plant injury is particularly more intense following dry periods, winters with low temperatures or late frosts [Treshow and Anderson (1989)]. Over much of Europe in the 1970s and early 1980s, drought conditions were quite noticeable [Kelly (1989)]. Drought has been shown to be responsible for damaging the fine roots of Douglas fir that can inhibit nutrient uptake [Marshall (1986)]. Thus periods of drought with episodes of various atmospheric pollutants such as ozone, the oxides of nitrogen (NO_x), SO_2 may have weakened the trees and predisposed them to other stresses [Nihlgard (1985), Rose (1985)].

The stresses involved can be categorised into three classes namely, predisposing, inciting and contributing [Ling and Ashmore (1987)]. Any one of these stresses may play more than one role, depending on the situation under which it is acting. Predisposing stresses generally have a longer-term role in weakening the tree and making it more susceptible to the shorter-term inciting factors. Inciting factors are sudden physical shocks predisposed by episodic stresses that increase the tree's susceptibility to secondary biotic stress, either by making it more attractive to insect attack or decreasing the ability of the tree to recover from the stress [Ling and Ashmore (1987)].

Once a tree has begun to decline, contributing factors, which are generally biotic stresses, may in time kill or weaken the tree by accelerating the decline (Table 1.1). These factors are often blamed for the condition of the tree, but in fact they have exerted their effect as secondary or tertiary stresses [McLaughlin (1985].

Table 1.1 Factors influencing forest decline [McLaughlin (1985)]

	Predisposing Stress	Inciting Stress	Contributing Factors
Functional Role	Chronic weakening	Triggering Episodes	Accelerators
Stressing agents	Climate Soil moisture Host genotype Soil nutrients Air pollution Competition	Insect defoliation Frost Drought Salt Air pollution Mechanical injury	Bark beetles Canker fungi Viruses Root-decay fungi Competition

Despite a recovering of the forest stands in the late 80's, recent work has brought to light that acid rain is still a problem. Bernhard Ulrich of the University of Gottingen has calculated how acid emissions need to be reduced if forest soils are to recover. He concluded that there needs to be a reduction of 60 to 76% (of 1982) levels immediately, with a further cut to 80% in the long term [Pearce (1990), Ulrich (1990)].

1.3 The Effects of Ozone Upon Plant Growth.

Ozone was implicated as a possible cause of forest decline in Europe by Arndt et al (1982) and Prinz et al (1982). Ozone is of major concern and its effects have been extensively modelled to try and determine its effects upon the environment [Lurmann et al (1984)]. Elevated levels of ozone have been known to occur over much of Europe and North America since the 1960s [Knabe et al (1973), Chevone and Linzon (1988)].

In Germany, the decline has been greatest at higher altitudes (600-800 m), and at these altitudes the ozone levels have also been found to be elevated. Ozone has been shown to cause an overall decrease in the net rate of photosynthesis [Myhre et al (1988), Keller and Matyssek (1990)]. A decrease in the rate of photosynthesis can lead to a decline in the rate of growth of a plant. If photosynthesis is decreased within the plant then it may use up its carbohydrate reserves too quickly. Ozone has been implicated in damaging the waxy cuticle of plants [Percey et al (1990)]. The epicuticular wax is thought to play an important role in a tree's winter hardiness and drought resistance [Percey and Baker (1990)]. Changes in the waxy cuticle structure and composition have been reported in areas where there is air pollution [Percey and Riding (1978), Sauter and Voss (1986)].

In various controlled fumigation experiments, ozone has been shown to produce a chlorotic mottling effect on Scots pine [Skeffington and Roberts (1985) and it has also been shown to increase injury in pea plants due to freezing [Barnes et al (1988)]. There is also evidence to suggest that ozone may delay the hardening process in spruce for winter. Such a failure of the leaf cells to develop protection against the cold would be especially serious in years of sudden temperature drops [Treshow and Anderson (1989)].

1.4 The Effects of Acid Rain on Plant Growth.

The term 'acid rain' was first used by Robert Angus Smith in 1872 to describe the rain falling around Manchester, Great Britain [Rose (1985)]. Acid rain is due to the oxides of sulphur and nitrogen being oxidised in atmospheric water droplets in the atmosphere to sulphuric and nitric acids [Bunce (1990)] and these can be deposited at great distances from their source [Cape and Unsworth (1987)].

The changing acidity of air and precipitation over North America and much of Europe has been known for some years. Large scale acidification of soils in Germany were

recorded between the 1920s and the 1980s However rain in Germany had reached a value of pH 4.2 by 1940. Rainwater has a pH value of approximately 5.6 [Treshow and Anderson (1989)]. The effect of acid rain and drought stress interaction on the yield of a corn cultivar showed that rainfall at pH 3.0 combined with a severe drought decreased the crop yield significantly as compared to the control that was provided with a rain of pH 5.6 [Banwart (1988)].

When forest decline first appeared in Germany, Bernhard Ulrich and his colleagues who were studying aluminium toxicity and nutrient cycling, proposed that the acidification of soils was accelerated as a result of deposition of acidifying compounds from the atmosphere. It was proposed that a decrease in pH, would result in aluminium and other ions present in the soil becoming soluble and hence more mobile [Fry and Cooke (1987), Treshow and Anderson (1989)]. The increased levels of aluminium in the soil were considered to be toxic and damaging to the fine root systems and to plants in general [Van Praag and Weissen (1985), Fry and Cooke (1987),Treshow and Anderson (1989)].

Since an increase in soil acidity results in an increase in the mobility of other ions, then 'heavy metals' can be made more mobile and thus be taken up into plants. 'Heavy metals' such as cadmium, mercury, nickel, zinc and copper have had their effects upon plant growth extensively studied. They are all detrimental to the well being of plants at various concentrations and their effects are well documented in the literature [Chaudry and Loneragan (1972), Cutler and Rains (1974), Krause and Kaiser (1977), Godbold et al 1985), Burton et al (1986)]. In soil once these toxic elements are mobilised they may become more subject to leaching from soils as well as being taken up into the plants' roots and foliage [Lepp (1981), Treshow and Anderson (1989)].

Nutrient deficiencies may arise due to acid rain induced leaching from the foliage. The leaching from foliage is believed to be aggravated by acid rain disrupting the cuticle

[Percey and Riding (1978), Sauter and Voss (1986), Percey and Baker (1990)] and cell membrane [Treshow and Anderson (1989)] and is further increased by interaction with gaseous pollutants [Zottl and Huettl (1986), Schatzle et al (1990)]. Magnesium was considered to be especially sensitive to leaching. In the Bavarian Forest magnesium concentrations in needles of declining spruce trees growing at altitudes above 1000m were found to be extremely low [Treshow and Anderson (1989)]. The trees exhibited the deficiency symptoms associated with magnesium deficiency, that is the yellowing and shedding of older needles and decreased growth. The deficiency of magnesium could lead to decreased frost hardiness of the needles , thus exacerbating the decline. It must be stressed that magnesium deficiency symptoms are not always prominent in declining areas, so other causes must also be sought [Treshow and Anderson (1989)].

1.5 Biological Indicators and Monitors.

The terms indicators and monitors have often been used in the context of employing biological materials such as plants in environmental studies. The use of the word indicator suggests the ability of the organism involved to simply indicate the presence or absence of a particular factor e.g. 'heavy metals'. In some cases, modifications of an individual plant such as chlorosis may indicate the presence of certain chemicals or an excess or deficiency of some elements [Martin and Coughtrey (1982)]. If a tree is deficient in nitrogen, chlorosis is apparent in the whole of the live crown, whereas in potassium deficient trees, chlorosis is confined to the older needles [Binns et al (1980)].

Plants have been used as indicators of gaseous air pollutants. It is known that the absorption of SO_2 can cause an increase in the acidity of aqueous solutions. Providing the dose of SO_2 is not acute, then an increase in the buffering capacity can be demonstrated [Koziol (1984)]. There are reports of decrease in the buffering capacity after exposure of plants to SO_2 and acid mists [Cape et al (1988)]. Sesame has been

found to be a sensitive indicator for SO_2 [Shu-Wen (1984)]. The plant *Nicotiana tabacum* is a sensitive indicator for ozone. It has a threshold for ozone injury of about 0.05 ppm, and it has been used in the monitoring of ozone levels over large geographical areas [Bell (1984)]. Other plant species that have been used as markers for the damage by photochemical oxidants to vegetation include morning glory, peanut and taro [Furukawa (1984)].

Enzyme assays have also been used as indicators of pollutant stress upon plants. A number of these assays have been used as diagnostic tests for air pollutants. Some enzyme systems that have been examined include peroxidase, polyphenoloxidase glutamate dehydrogenase [Darrall and Jager (1984)] and super-oxide dismutase [Tanaka et al (1980), Ostrovskaya et al (1990)]. For example, the peroxidase enzyme system of plants has been shown to be affected by SO_2 [Keller and Scwanger (1971), Huttenen (1981)] and by viral infection [Baaziz and Saaidi (1988)]. Also peroxidase activity was found to be elevated after exposure to SO_2 [Declare et al (1984)]. Ozone and road traffic fumes have also been reported to cause elevated activities in enzyme systems [Darrall and Jager (1984)]. Peroxidase activity is known to increase in plant cells under various stress conditions, such as when affected by toxic gases, mechanical injuries to plant parts or attack by parasites [Declare et al (1984), Darrall and Jager (1984), Spanu and Bonfante-Fasolo (1988)]. It has been suggested that an increase in the activity of enzymes in plants that are under stress may be in direct response to the destruction of the cell membrane [Farkas et al (1964)]. Increase in peroxide activity when they have been subjected to SO_2 may be due to the conversion of sulphite to sulphate by hydrogen peroxide in the presence of peroxidase [Fridovich and Handler (1961)].

Hydrocarbon emissions from the needles of conifers have been used as markers for stress and injury in trees. Ethylene, produced by all plants is widely known as a stress

indicator in living cells, its production increasing in proportion to the degree of stress [Cape et al (1988)].

1.6 Possible Local Sources of Pollution.

The decline in growth at the Afan 1 Forest sites may be due to pollution from local industry both past and present, including iron and steel works such as the one at Port Talbot. This is approximately 20 Km from the Afan 1 Forest. Petrochemical industry (again Port Talbot), coke ovens, coal spills (which contain various 'heavy metals') and power stations [Welsh Office (1975)] have also been implicated as sources of pollution [Rose (1985)] It is known that these industries contribute large quantities of pollutants to the environment in the forms of smoke, dust and gases [Hutchinson and Whitby (1974)]. These pollutants include the oxides of sulphur and nitrogen, ozone and heavy metals [Fangmeier et al (1990)], which on deposition can be injurious to plants [Krause and Kaiser (1977), Koziol and Cowling (1980)]. Sulphur oxides can damage the unsaturated fatty acids within plants. Since these fatty acids play an important role in the cell membrane then any changes to their composition can have catastrophic effects upon the cells [Southerland et al (1982)].

Work has shown that there are elevated levels of Cd, Cu, Ni and Pb in oak leaves, grasses and soils in the Rhondda Fawr valley (South Wales) especially in the more urbanised areas [Burton and John (1977)]. This fact of elevated 'heavy metals' within the urban areas supports the view that motor vehicle emissions and coal burning can be major sources of heavy metals under the right conditions.

Chlorosis in plants can be an indicator of 'heavy metal' contamination. This has to be established by taking samples and determining the metal content. Moss bags have been successfully employed to determine the airborne deposition of 'heavy metals'. This

method was used by Goodman and Roberts (1971) in determining the heavy metal burdens of the Swansea and Neath Valleys. Their results showed the levels of Cd, Cu, Ni and Pb to be much higher than the relatively non-industrialised regions to the west. It was found from their results that there was a similar geographical trend for all the metals in that their respective levels decreased with increasing distance from the source.

A report on the possible causes of poor growth of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) at a forest near Margam (South Wales) stated that the soils were amongst the most infertile in the principality [Jones (1970)]. This study tried to relate the distribution of industrial hazards such as the levels of sulphur dioxide, salt and temperature fluctuations to the extent of tree damage. It was suggested that a combination of factors may be the cause of the poor growth.

1.7 Focus of Study.

Foliar tissue is the focal point for many of the plant's functions and it is a fairly sensitive indicator for elements such as potassium and nitrogen that are directly associated with photosynthesis and chlorophyll. The material is convenient and relatively easy to obtain as a sample. It has been used in extensive surveys for indications of forest decline throughout Europe [Cape et al (1988)]. The foliar tissue can also be the focal point for various airborne pollutants as mentioned in sections 1.3, 1.4 and 1.6.

Chlorophyll levels have been used as an indicator of the upper critical tissue of cadmium when chlorosis has been used as an observable effect of cadmium toxicity [Burton et al (1986)] in wheat. If chlorosis is occurring it may be worth considering the foliar levels of chlorophyll, since this substance is essential for photosynthesis and hence, ultimately, carbohydrate production [Stryer (1988)]. Any decrease in the chlorophyll content may be detrimental to the plant's well being. The productivity of a plant is related to the amount

of chlorophyll present [Emberlin (1983)]. Therefore measurements of the amount present may give indications of the health of the tissue and hence the plant.

The mineral contents of leaves or needles are usually used to determine the nutritional status of the plant, The results of which can be used to achieve early diagnosis of latent or already established pollution damage. Unfortunately many parameters influence the mineral content of leaves and needles, examples being the type of soil, species of plant, genotype, age of the plant, and the position of the leaf or needle on the tree [Bouma (1983), Krivan and Schaldach (1986)]. The Forestry Commission regularly analyses the foliar levels of nitrogen, phosphorus, potassium, magnesium, sulphur and calcium [Binns (1980), Coutts (Pers. Comm.) (1986)]. If the results obtained suggests a deficiency in one or more of these elements, then the use of appropriate fertilisers may be used to reverse the effects [Binns et al (1980)].

Since various 'heavy metals' have a detrimental effect on the levels of chlorophyll [Morgan (1983), Burton et al (1986)], it may be worth examining the foliar levels of certain heavy metals. The methods of examining the metals, that are by atomic absorption/emission techniques are destructive in that the tissue has to be digested. The examining of total foliar metal concentrations can have its drawbacks. It does not give any indication as to where the highest levels of the metals are to be found within leaves or needles. Also the method does not give indications to leaf surface metal levels.

Foliar analysis is more convenient than analysis of the roots from trees in their natural environment. Analysis of metals in roots would be more difficult than analysing for the same metals in the foliage due to the problem of soil debris associated with root systems. This has to be thoroughly washed off or inaccurate results may ensue. Also, digging up

roots from trees may lead to other problems such as the root systems getting damaged due to careless digging [Morgan (Pers. Comm). (1986)].

The foliar metals examined in this study are potassium, calcium, magnesium, copper, cadmium, nickel and zinc. Potassium, calcium and magnesium are essential for plant growth and as such important to the well being of a plant. Deficiencies of these elements can lead to a retardation of growth [Binns et al (1980), Mengel and Kirkby (1981)]. Copper, zinc and possibly to some extent nickel is important to the well being of plants, but are not usually required in high concentrations [Hutchinson (1981), Lepp (1981), Page et al (1981)].

The toxic effect of nickel upon plants was first discussed in 1893 by Haselhoff [Hutchinson (1981)]. Since then there have been many reports on the toxicity of nickel on plants. Some studies have shown that toxic levels of nickel have caused poor growth and chlorosis. The levels of nickel in plants growing in uncontaminated soils have nickel levels reported to be below 10 ppm [Hutchinson (1981)].

Copper was first shown to be an essential element to plant growth in 1931 [Sommer (1931)]. Since then it has been shown that many enzyme systems require copper as a co-factor [Phipps (1975)]. Copper deficiency can lead to reduction in the amount of foliage whereas an excess can cause chlorosis and a stunting of growth [Foy et al (1978), Morgan (1983)].

Cadmium is a well-known toxic element. Its uptake into a plant may be related to soil pH, since it has been shown that as the soil pH increases, the concentration of cadmium within the plant decreases [Bingham et al (1980)]. Cadmium can also cause acute chlorosis and retardation of Sitka spruce growth [Morgan (1983), Burton et al (1986)]. It

has been shown that the natural concentration of cadmium within plants varies according to species [Page et al (1981)]. Zinc has been found to be important in the function of some enzymes [Collins (1981)]. Zinc is also known to form stable complexes with DNA and RNA [Collins (1981)]. If there is not an adequate supply of zinc to a plant, then chlorosis and stunted growth may ensue [Collins (1981)]. Zinc deficient plants may show a reduced level of zinc in their foliar tissues, usually less than 15 ppm dry weight whereas healthy plants have zinc in the range of 10-100 ppm depending on the species [Collins (1981)]. Zinc in excess is known to be toxic to plants, and the usual symptoms are a retardation of growth and chlorosis [Collins (1981)]. Zinc, Cu and Cd concentrations found in the xylem rings of Scots pine have been correlated to their concentrations in the soil [Lukaszewski et al (1988)].

1.8 Study Aims.

In general the aim of this study was to establish whether certain biological indicators such as chlorophyll levels, and some foliar metals together with certain enzyme systems are an effective tool for monitoring tree growth. The Forestry Commission supplied data on tree growth and soil-solution elemental analysis [performed by the Central Electricity Generating Board (1986)]. This could be used as a data base in an attempt to establish relationships with foliar data. Therefore sampling of the foliage in Sitka spruce (*Picea sitchensis* (Bong) Carr.) was carried out in 1986 and 1987 and analysed for chlorophyll, and selected metals and nutrients.

Sitka spruce was chosen as it is one of the most widely planted species in South Wales. It grows relatively well on damp sites, especially high land, and it withstand exposure better than most conifers. It is an important tree to the Forestry Commission since its timber is widely used for pulping and it finds many uses in the building industry [Forestry Practice (1978)].

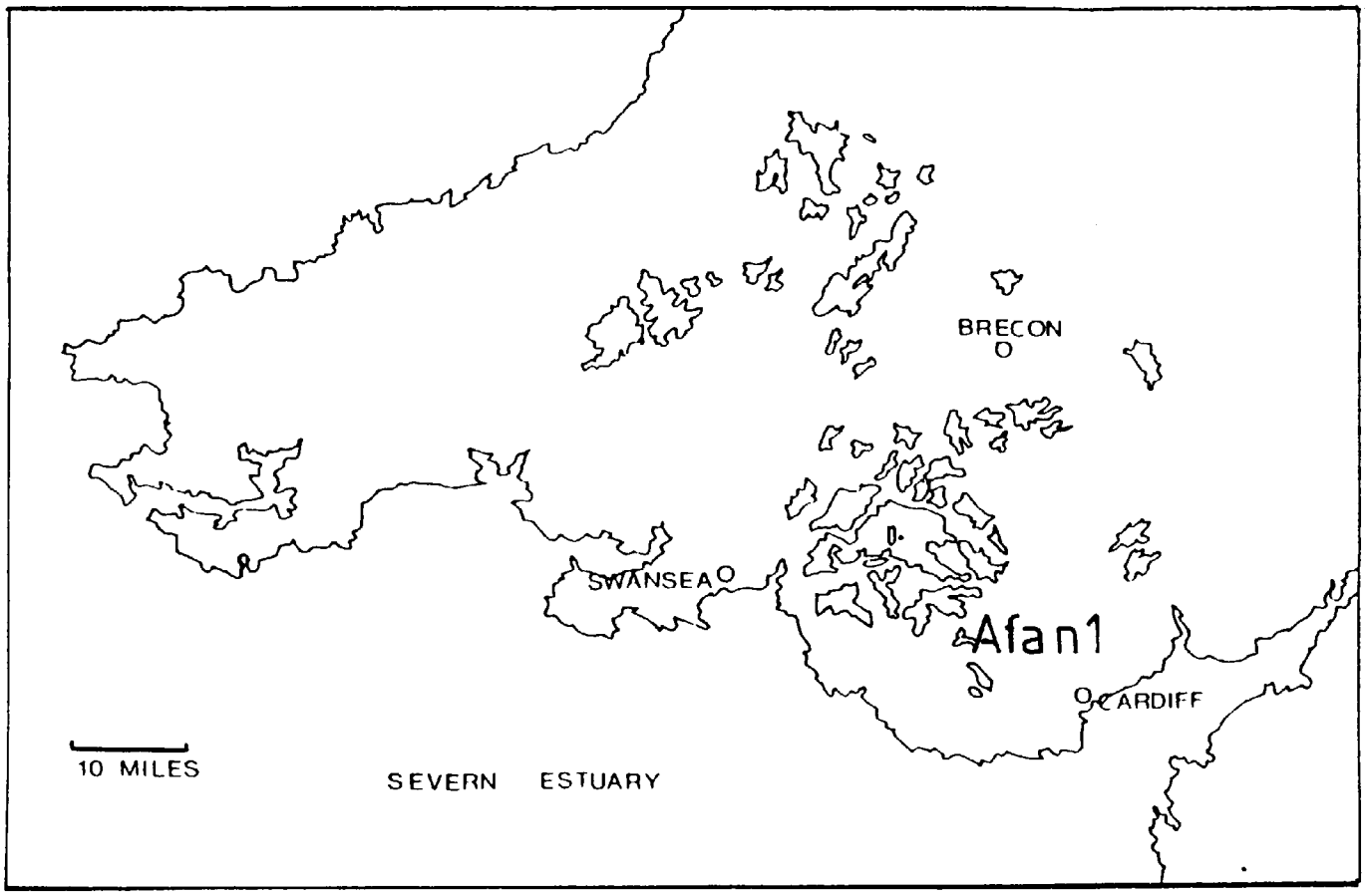
The quantification of chlorophyll levels in coniferous trees can be difficult and is often inaccurate. Historically a set of equations for spectrophotometry has been used [SCOR-UNESCO (1964)]. More modern methods have now been employed for the routine analysis of chlorophylls such as High Performance Liquid Chromatography (HPLC). One objective of this study was to validate the SCOR-UNESCO equations using the more accurate HPLC method so that correction factors could be determined and if necessary to allow rapid and accurate spectrophotometric determinations. Following on from this, it was decided to try and develop a method for analysing chlorophyll levels via Supercritical Fluid Chromatography (SFC). This method promises faster analytical times.

Anomalies in growth parameters have often been observed within sites of pairs of trees located next to one another. An objective of this study was to establish whether there are any differences in the chlorophyll content, nutrient and trace metal levels that could account for the differences in growth. Finally experimental work using two years old Sitka spruce seedling was carried out with the objective of determining the main effects and possible interactions of cadmium and copper upon the growth, chlorophyll and nutrient levels.

Soil elemental analysis can prove to be difficult and time consuming. The variability of the soil properties at a particular site plays an important factor in deciding how many replicate samples should be taken. It has been shown that there is little advantage to be gained from sampling sites of one hectare as compared with 0.01 hectare [Blythe and Macleod (1978)]. Sites of 0.01 hectare have been sampled and the results obtained have been used in correlation's with tree growth parameters [James et al (1978)]. Soil is usually removed from the top 0-15 centimetre for analysis.

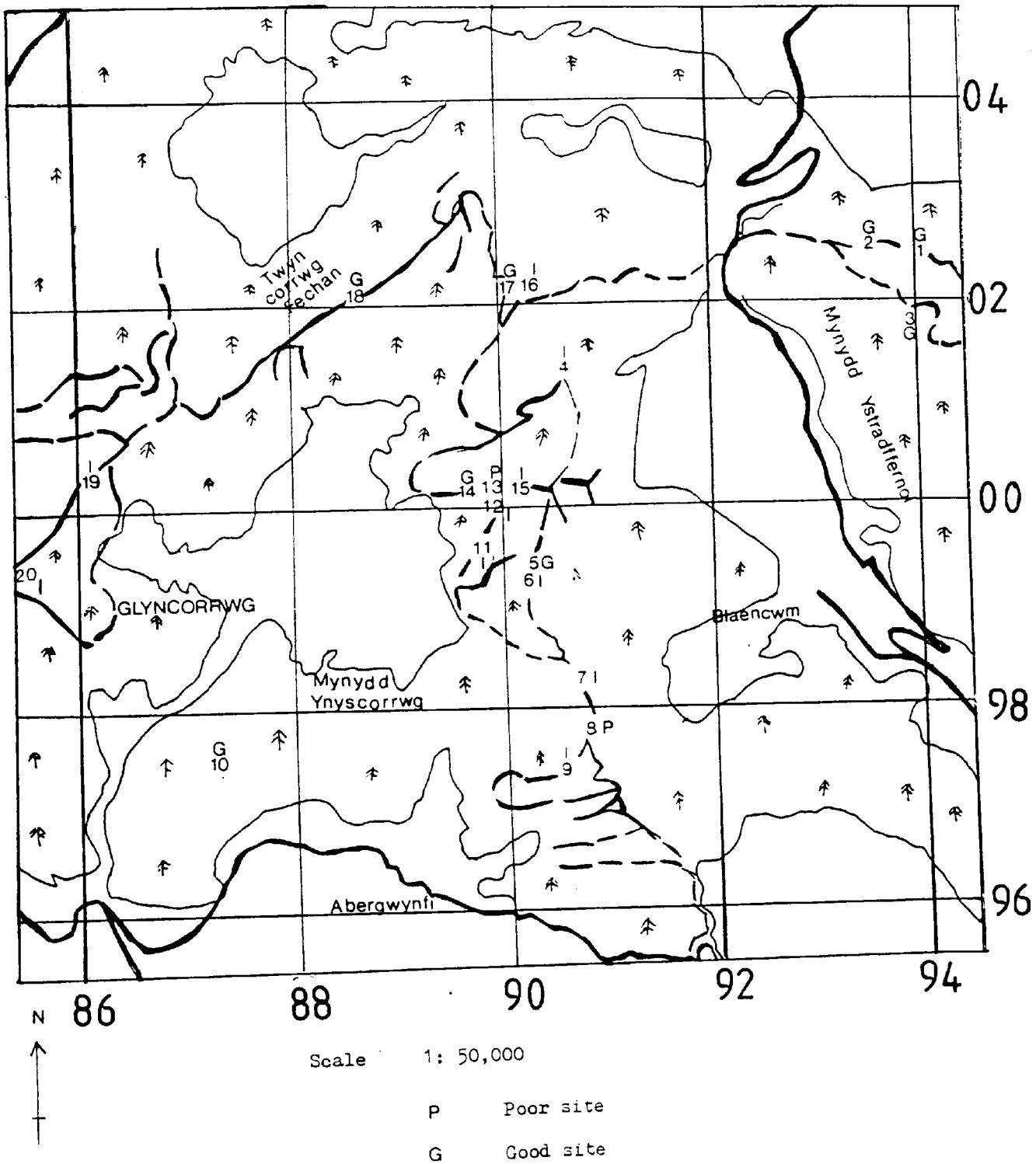
Since soil properties and mineral content vary greatly within selected plots, it is often difficult to assess the significance of the results of an analysis [Ball and Williams (1968)]. The number of samples taken must be sufficient to bring the variation about a mean value to a statistically reliable value. It has been shown that the average number of top soil samples required per plot to secure 95% confidence limits for a range about a mean was twenty-nine samples for 0.5M acetic acid extractable nutrients [Morgan (1983)]. Soil elemental analysis was not carried out at the nineteen Afan 1 forest sites due to the high number of replicates that would be required to obtain meaningful results that is, the facilities that were available could not handle the large numbers of samples and the equipment used for analysis did not have the necessary sensitivity.

Fig. 1.1 Map of South Wales and Part of Western England.



Forests

Fig. 1.2. Map of the Forest Sites at the Afan 1 Forest.



CHAPTER 2

2. 1986 FOLIAR ANALYSIS.

2.1 Brief Description of the Sites at the Afan 1 Forest.

There are twenty sites of Sitka spruce at the Afan 1 Forest that are being monitored by the Forestry Commission. Of these 20, sites 1 to 18 and site 20 were studied in this project. Site 19 was omitted since it had low growth parameter measurements. The Forestry Commission selected the sites so that differences in age alone were unlikely to cause the observed differences in growth at these sites.

There is much evidence of damage at many of the sites in the Afan 1 Forest. For example, site 4 has a low growth increment (17 cm/annum). The soil is a deep peat type. The surface has many drainage ditches but despite this, the peat still retains quite a lot of water. There is a considerable amount of 'bent-top' at this site with epicormic shoots being quite prevalent. Many of the trees have a yellow flecking. On their needles, which may be retained for a number of years [Coutts (Pers. Comm.) (1986)]. As at many of the poor sites, site 4 has many healthy trees, but some of them have epicormic growth together with a degree of chlorotic mottling. It was found that potted trees placed on a tower developed flecking. As a result this flecking may be of climatic origin rather than due to the soil conditions.

Site 8 has a fairly high growth increment (50 cm/annum). It has good drainage compared to site 4. The bent-top, flecking and chlorosis that is very evident at site 4, only occurs to a lesser degree.

At site 9, the symptoms of die-back are quite evident. It has a low growth increment (21 cm/annum). The soil is well drained. Some of the trees possess only epicormic growth with no other normally developing foliage and there is a large degree of bent-top.

Site 14 has a growth increment of 71 cm/annum. The trees at this site are quite healthy with very few of the poor growth symptoms being discernible. The soil is also well drained. Site 15 is adjacent to Site 14, and is in marked contrast to that site. Site 15 has a low growth increment (15 cm/annum). The soil is poorly drained and is in part due to an 'iron-pan' type soil being present that prevents drainage. The foliage is quite poor with bent-top being noticeable.

2.2 Sampling.

At each site there are eight trees that the Forestry Commission has marked out for their own particular survey. All trees at the sites at Sitka spruce (*Picea sitchensis* (Bong.) Carr.). One tree was picked randomly at each site for this survey. The foliar sampling method used in this study was a standard Forestry Commission technique [Coutts (Pers. Comm.) (1986)]. Branches were removed from the third whorl down from the apical shoot with the use of a forty feet long pole with a cutter attached at one end. Samples with similar aspects were removed wherever possible.

The sampling was performed in late October/early November 1986. It is thought that sampling performed during the Autumn/Winter is best since these are periods where there is little physiological change within the plant. Sampling during such a stable period

has the advantage that comparisons between different stands are quite valid even though times of sampling may vary [Morgan (1983)]. For the analysis, the three growth years present on the branch were examined, these being the year 1 (present growth year), year 2 and year 3 (oldest) needles.

2.3 Elemental Analysis of Foliar Tissues.

The needles were carefully cut from the branches and the waxy cuticle removed using a suitable organic solvent (carbon tetrachloride).The needles were dried overnight at 105^{o}C. They were then ground using a pestle and mortar and the samples were then bagged. The bags were then shaken and 0.5 g duplicates of the powdered needles were removed from the bags and digested with 10 cm^3 of a 3:1 mixture of concentrated nitric and perchloric acids (both AnalaR grades). The boiling tubes were heated to complete the dissolution of the plant material. After being allowed to cool, the digests were filtered through Whatman No. 42 paper and diluted to 10 cm^3. The concentrations were determined by atomic absorption spectrophotometry (AAS) via a Varian 1275 atomic absorption spectrophotometer and flame photometry (Corning 400) using appropriate standards and blanks. The concentrations of the metals were calculated directly from the Varian 1275 linear calibration facility. The conditions for the analysis are given in Table 2.1 and Table 2.2. The results of the analysis are given in Table 2.3.

Table 2.1. Analytical Data for the Elements Determined by AAS.

Element	Wavelength (ηm)	Spectral Band Width ηm)	Optimum Working range mg dm^{-3}	Sensitivity mg dm^{-3}	Detection Limit mg dm^{-3}
Cd	228.8	0.5	0.5-2.00	0.011	0.006
Cu	324.7	0.2	2.0-8.00	0.004	0.003
Ni	232.0	0.2	3.0-12.0	0.066	0.008
Zn	213.9	0.2	0.4-1.20	0.009	0.02
Mg	202.5	1.0	5.0-20.0	0.090	

All elements were determined using a gas mixture of air and acetylene. The sensitivity is defined as the concentration of that element in aqueous solution that absorbs 1% of the incident radiation intensity passing through a cloud of atoms that is being determined. The detection limit is defined as the concentration in solution of an element that can be detected with a 95% certainty, i.e. the quantity that gives a reading equal to 1.64 times the standard deviation of a series of at least 10 determinations at near blank level [Morgan (1983)].

Table 2.2. Analytical Data for the Elements Determined by Flame Photometry

Element	Optimum Working range mg dm^{-3}
K	3-100 (full scale deflection)
Ca	5-100 (full scale deflection)

Table 2.3. 1986 Foliar Analysis (mg kg^{-1} dry weight)

Site No.	K yr1	K yr2	K yr3	Ca yr1	Ca yr2	Ca yr3	Mg yr1	Mg yr2	Mg yr3
1	4213	6000	4571	3652	4326	2204	1043	522	416
2	5520	5918	4899	4720	5959	4980	1128	898	677
3	6653	4560	4400	1997	2240	1760	858	584	478
4	5125	2000	2612	2847	2640	5061	1079	608	996
5	5405	4560	4244	1856	2320	2922	1073	832	501
6	5551	3177	3760	1674	2039	3840	1029	760	984
7	4941	4235	MD	1529	2509	MD	690	651	MD
8	5255	4706	3529	2745	3255	3686	815	611	533
9	4960	3184	2867	1923	1714	2870	1083	482	815
10	5961	5520	4735	1922	1950	1143	1231	880	531
11	4941	3882	2245	1372	2039	1306	973	753	416
12	4313	3614	2720	1608	2227	3240	1615	758	1368
13	6000	4160	MD	1184	1120	MD	988	766	MD
14	5696	5800	4424	3560	5440	2922	1031	720	501
15	4078	3400	2867	1294	2000	2870	965	1224	815
16	4560	2824	2240	1280	1019	1440	1072	580	760
17	6204	5040	3059	5469	7280	3686	1032	640	408
18	6118	6400	4880	4706	4880	3760	898	640	496
20	5880	5117	2960	2960	2824	1520	1512	1160	648

Site No.	Cd yr. 1	Cd yr. 2	Cd yr. 3	Ni yr. 1	Ni yr. 2	Ni yr. 3	Cu yr. 1	Cu yr. 2	Cu yr. 3
1	0.0	0.0	1.1	12.2	2.0	6.1	1.0	1.2	5.5
2	0.0	0.5	0.0	4.0	6.1	6.1	1.6	3.1	4.3
3	30.2	0.6	0.5	6.4	3.0	4.1	1.9	2.0	5.6
4	0.8	1.2	1.0	5	6.0	7.7	2.4	2.4	4.6
5	0.2	0.0	0.7	1.8	6.0	4.0	4.9	2.6	4.5
6	0.6	0.2	0.5	2.7	4.9	4.6	5.3	1.2	2.8
7	0.8	0.5	MD	5.9	0.0	MD	3.1	2.4	MD
8	0.4	0.5	0.9	11.4	5.9	5.9	0.4	3.9	3.9
9	0.8	1.0	0.7	4.5	6.1	4.6	2.4	0.2	3.3
10	27.8	0.0	1.0	9.2	6.0	10.2	1.7	2.6	3.3
11	6.0	0.0	0.7	3.9	2.9	6.1	2.6	1.8	2.9
12	0.2	0.2	0.0	5.9	0.5	6.0	1.2	2.2	2.6
13	12.3	0.4	MD	8.3	4.2	7.7	2.6	2.4	MD
14	0.6	1.0	0.7	8.0	6.0	7.7	4.2	3.6	4.5
15	1.2	0.7	0.7	0.0	4.0	4.6	2.2	3.4	3.3
16	2.4	0.9	0.7	3.9	2.0	4.0	1.6	1.6	2.5
17	38.4	0.5	0.5	14.6	0.0	7.8	5.4	2.6	5.1
18	1.4	3.2	1.0	10.0	4.2	8.0	0.4	2.2	3.4
20	0.8	0.2	1.0	1.8	2.00	2.0	2.6	2.8	3.8

0.00 = below detectable limit
MD = missing data (due to this growth year not being present)

2.4 Chlorophyll Determination.

The samples were analysed for chlorophyll content on the day they were taken to avoid any possibility of degradation of the chlorophyll. The needles were carefully removed from the branches and then for each growth year were bagged and shaken. Duplicate 0.5 g of fresh Sitka spruce needles were macerated in a chilled pestle and mortar using a little acid washed sand (40-100 mesh) using ice-cold 90% aqueous acetone. The macerate was then centrifuged for 3 minutes at 4500 rpm. The supernatant was decanted and the pellet resuspended in 90% aqueous acetone and re-ground. This was also centrifuged, the supernatant was then combined with the previous supernatant, and the volume made up to 25 cm^3. The concentrations of the chlorophylls were determined using the equations produced by the SCOR-UNESCO Working group 17 (1964) (Table 2.4). Absorbances were obtained on a Perkin-Elmer Lambda 3 spectrophotometer. The results for the 1986 foliar chlorophyll levels are given in Table 2.5

Table 2.4. SCOR-UNESCO Equations for Determining Chlorophyll 'a' and Chlorophyll 'b' Concentrations in Plant Tissue (Fresh Weight).

$$\text{Chl a } (\text{mg dm}^{-3}) = 11.64\,(A663) - 2.16(A645) + 0.10\,(A630)$$

$$\text{Chl b } (\text{mg dm}^{-3}) = -3.96\,(A663) + 20.97\,(A645) - 3.66\,(A630)$$

$$\text{Chl c } (\text{mg dm}^{-3}) = -5.53\,(A663) - 14.81\,(A645) + 54.22\,(A630)$$

where A = the absorbances at 663ηm, 645ηm and 630ηm minus the absorbance at 750ηm (background correction).

Table 2.5. 1986 Foliar Analysis Chlorophyll Levels (mg kg^{-1} fresh weight)

Site No.	Chl a yr.1	Chl a yr.2	Chl a yr.3	Chl b yr.1	Chl b yr.2	Chl b yr.3	a/b yr.1	a/b yr.2	a/b yr.3
1	614	1303	915	229	561	362	2.68	2.32	2.53
2	1444	1123	1178	420	377	390	3.44	2.98	3.02
3	1235	1294	1111	441	526	334	2.80	2.46	3.33
4	718	1621	928	230	581	326	3.12	2.79	2.85
5	1087	1373	1471	399	542	548	2.72	2.53	2.60
6	1091	1217	1080	314	264	368	3.47	4.61	2.93
7	879	1354	MD	236	468	MD	3.72	2.89	MD
8	1383	1500	2875	460	633	1223	3.01	2.37	2.35
9	572	573	913	160	183	298	3.56	3.13	3.06
10	1062	1299	1603	303	377	529	3.50	3.45	3.03
11	1264	1083	1092	420	352	398	3.01	3.08	2.74
12	1300	1370	1272	426	452	482	3.05	3.03	2.64
13	850	974	MD	234	313	MD	3.63	3.11	MD
14	1017	1371	1459	275	501	567	3.70	2.91	2.57
15	556	767	1003	132	216	295	4.21	3.55	3.40
16	645	1182	815	199	404	303	3.24	2.93	2.69
17	1290	2510	1167	396	822	431	3.26	3.05	2.71
18	1103	1654	1154	341	545	404	3.23	3.03	2.86
20	1521	1931	1326	502	623	504	3.03	3.10	2.63

MD = missing data (due to this growth year not being present)

2.5 Growth Parameter Data Supplied by the Forestry Commission.

This information is provided in Table 2.6. The Forestry Commission measures tree growth potential using a yield class system. The growth of trees may be quantified in terms of increases in height, weight, diameter, volume or dry matter - of this, only height, diameter and volume are easily measured and the most useful is volume. Measurable volume is defined as stem-wood having at least 7 cm diameter overbark [Edwards (1981)]. Current Annual Increment (CAI) represents the annual volume increment (m^3 ha^{-1}), Mean Annual Increment (MAI) represents the average annual increment from planting. General Yield Class (GYC) is obtained from the top height and the age of the stand.

Table 2.6 Growth Parameter Data at the Afan 1 Forest Site

Site No.	Tree Height (m)	CAI m/yr.	MAI m/yr.	GYC	PGYC	Date of Planting
1	7.41	0.72	0.44	14	18	1968
2	6.71	0.72	0.39	12	20	1968
3	3.81	0.55	0.50	14	16	1974
4	3.95	0.17	0.16	4	2	1963
5	5.74	0.74	0.34	12	20	1968
6	3.45	0.16	0.20	6	2	1968
7	4.71	0.30	0.10	6	2	1960
8	6.83	0.30	0.27	8	10	1960
9	5.06	0.21	0.23	6	2	1963
10	11.05	0.47	0.41	12	10	1958
11	4.15	0.27	0.24	8	2	1968
12	3.68	0.33	0.22	8	4	1968
13	3.68	0.82	0.28	12	22	1968
14	6.40	0.71	0.36	12	18	1960
15	8.95	0.21	0.21	8	2	1960
16	5.30	0.29	0.24	8	2	1963
17	8.01	0.66	0.38	12	16	1964
18	8.01	0.62	0.35	10	14	1962
20	4.35	0.19	0.21	6	2	1960

Ht = tree height CAI = Current Annual Increment
MAI = Mean Annual Increment
GYC= General Yield Class
PGYC = Present General Yield Class

2.6 Soil Solution Elemental Analysis.

This information on the sites at the Afan 1 Forest was provided by the Central Electricity Generating Board for the Forestry Commission in 1986, and is listed as supplied. Samples of soil solution were obtained by centrifugation and decantation and were then analysed by the CEGB using and ICPE (Inductively Coupled Plasma Emission Spectrometer).

Table 2.7. Soil solution Analysis Data Supplied for The Forestry Commission by the CEGB (1986) (mg dm^{-3})

Site No.	pH	Al	As	B	Ca	Cd	Cu	Fe	K
1	3.53	3.260	0.047	0.190	10.70	0.000	0.033	0.173	5.83
2	3.47	0.679	0.045	0.332	3.75	0.000	0.033	0.278	6.46
3	3.75	0.727	0.000	0.262	3.04	0.000	0.034	0.155	3.61
4	4.49	0.522	0.683	0.864	8.50	0.019	0.053	0.264	10.60
5	3.76	0.366	0.000	0.308	3.16	0.000	0.032	0.057	30.10
6	3.51	1.240	0.095	0.272	4.79	0.013	0.042	0.215	6.23
7	3.86	0.545	0.402	0.341	2.48	0.000	0.042	0.188	6.67
8	3.46	0.546	0.046	0.320	3.06	0.000	0.029	0.145	31.70
9	4.76	0.399	0.286	0.579	7.25	0.000	0.074	0.349	19.00
10	4.63	0.437	0.000	0.337	1.85	0.000	0.045	0.253	2.82
11	4.93	0.473	0.000	0.229	3.65	0.000	0.032	1.330	3.07
12	4.62	0.340	0.161	0.300	1.74	0.000	0.040	0.220	3.51
13	3.67	1.400	0.000	0.216	2.23	0.000	0.035	0.144	3.27
14	3.57	0.833	0.000	0.184	1.90	0.000	0.030	0.187	22.40
15	3.51	3.040	0.000	0.261	3.23	0.014	0.068	0.241	6.80
16	3.59	2.760	0.041	0.308	4.67	0.210	0.034	0.138	4.09
17	MD	MD	MD	MD	MD	MD	MD	MD	MD
18	MD	MD	MD	MD	MD	MD	MD	MD	MD
20	4.29	0.443	0.143	0.734	2.43	0.000	0.060	0.365	4.21

Site No.	Mg	Mn	Na	Ni	P	S	Si	Zn
1	2.000	0.047	7.01	0.000	0.246	3.50	3.090	0.498
2	1.550	0.014	10.50	0.000	0.000	8.75	1.560	0.855
3	1.510	0.022	7.37	0.018	0.000	2.50	0.983	0.590
4	2.190	0.029	18.70	0.019	0.693	11.80	2.050	1.770
5	1.280	0.021	7.83	0.000	0.288	3.46	2.770	0.781
6	3.020	0.041	8.27	0.015	0.000	4.47	2.550	0.896
7	0.988	0.019	8.30	0.000	0.000	5.60	2.180	0.777
8	1.070	0.203	8.19	0.000	0.309	4.27	3.760	0.703
9	0.849	0.017	14.00	0.018	0.332	7.54	1.830	1.070
10	0.202	0.003	6.46	0.000	0.422	4.71	3.430	0.477
11	1.350	0.809	9.62	0.037	0.311	8.68	3.030	0.741
12	0.469	0.004	7.15	0.016	0.000	4.94	1.400	0.527
13	1.210	0.079	7.26	0.016	0.000	2.12	2.400	0.528
14	1.100	0.020	6.68	0.000	0.000	3.49	2.890	0.472
15	2.280	0.212	9.11	0.028	0.000	4.10	3.150	0.772
16	2.800	0.050	10.40	0.042	0.000	3.97	2.260	1.150
17	MD	MD	MD	MD	MD	MD	MD	MD
18	MD	MD	MD	MD	MD	MD	MD	MD
20	1.110	0.010	16.30	0.000	0.275	12.30	1.490	1.380

MD = missing data (Not analysed by the CEGB)
0.000 below detectable limit

2.7 Data Analysis

The growth parameter data (Table 2.6) were subjected to three types of cluster analysis - Ward, Median and Centroid using the squared Euclidean distance as the distance measure, with the rescaled distance being a measure of the between the object distances [Norusis (1985)].

Cluster analysis (see Appendix 1) is an auto learning procedure that can provide valuable information on the groupings within a given data set. A fuller description of cluster analysis is to be found in Appendix 1. The results of the cluster analysis are depicted in the dendrograms of Figures 2.1, 2.2 and 2.3.

These dendrograms in each case show two distinct groups of trees:-

Class 1 (Good Growth): sites 1, 2, 3, 5, 10, 13, 14, 17 and 18

Class 2 (Poor Growth): sites 4, 6, 7, 8, 9, 11, 12, 15, 16 and 20

The Forestry Commission had classified the sites using the growth parameter data in the following manner:-

Class 1 (Good Growth): sites 1, 2, 3, 5, 10 ,14, 17 and 18

Class 2 (Poor Growth): sites 4, 6, 7, 9, 11, 12, 15, 16 and 20

Sites 8 and 13 were classified by the Forestry Commission as intermediate growth [Pers. Comm. (1986)]. The cluster analysis appears to concur quite well with the Forestry Commission's classification of the data.

Cluster Analysis Performed on the Forestry Commissions Growth Parameters.

Fig. 2.1 **Ward**

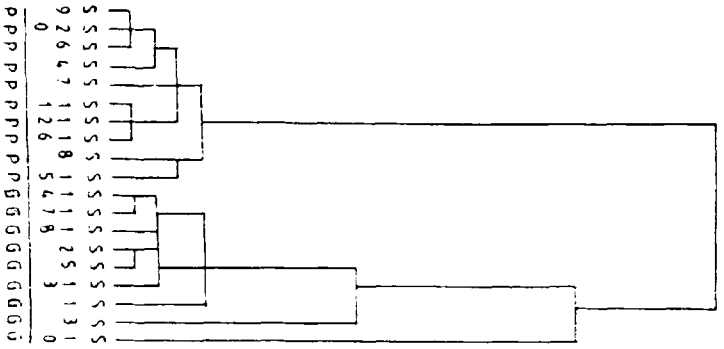


Fig 2.2 **Median**

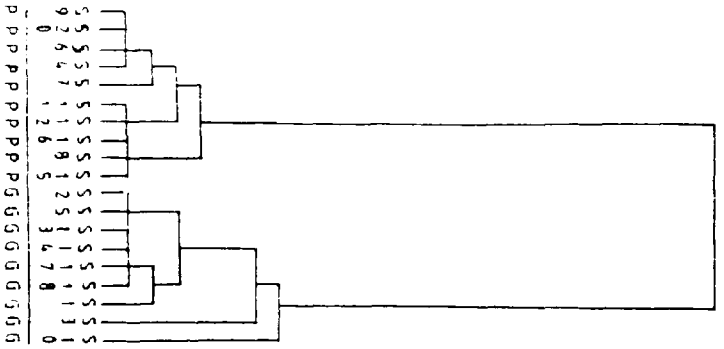
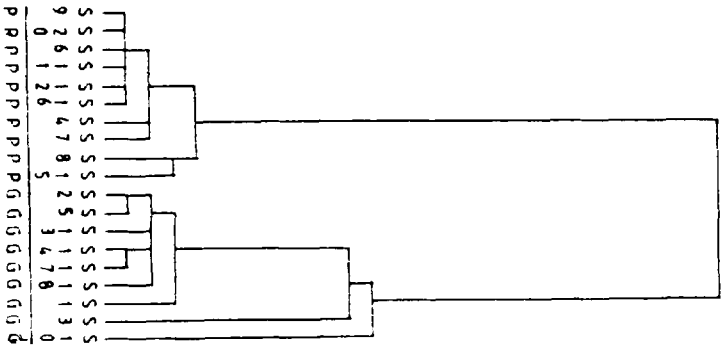


Fig 2.3 **Centroid**



P = Poor Growth

G = Good Growth

S = Site Number

The growth parameters for the good and poor growth sites at the Afan 1 Forest were found to be normally distributed (Table 1 Appendix 2). Student's t-test was therefore applied to the two tree classes, that is the good and poor growth as indicated by the cluster analysis. The results are shown in Table 2.8. For all the growth parameters there were significant differences at a probability of 99% or better. The good growth sites had significantly higher growth parameters. These parameters being measurements of how well a stand is growing, indicates the good growth sites to be doing better than the good growth sites.

Table 2.8. t-test Result for the Good and Poor Growth Classes

	CLASS 1 Good Growth		CLASS 2 Poor Growth		
	Mean	**sd**	**Mean**	**sd**	
Ht (m)	7.34	2.05	4.83	1.21	***
CAI (m year^{-1})	0.67	0.11	0.67	0.06	****
MAI (m year^{-1})	0.38	0.06	0.21	0.21	****
GYC	12.22	1.20	6.80	1.39	****
PGYC	17.11	3.62	3.00	2.54	****

Sig. Diff. **** 99.9%
*** 99.0%

Since the cluster analysis of the growth parameter data suggests the existence of poor and good growth sites, Principal Component Analysis (PCA) was applied to the 1986 foliar data to see if a similar pattern could be obtained across the sites. PCA can provide information on class structure and it will also give information on the involvement of variables in each principal component (PC). A description of PCA is given in Appendix 1.

The results also point to the existence of two distinct classes within the foliar data, thus confirming the result of the cluster analysis on the Forestry Commissions growth data. The PC score plots are shown in Figures 2.4 and 2.5 (Fig. 2.4 shows the distribution of the individual sites and Fig. 2.5 shows the distribution of the classes). They indicate that

Class 1 (Good Growth) consists of sites 1, 2, 3, 5, 10, 13, 14, 17 and 18 and Class 2 (Poor Growth) consists of sites 4, 6, 7, 8, 9, 11, 12, 15, 16 and 20. The PCA result of the foliar data corresponds well with that of the cluster analyses. Site 8, which is classed as an intermediate by the Forestry Commission appears to belong with the poor sites (Fig. 2.5).

The plots shown in Figures 2.4 and 2.5 are for the first two principal components (PCs). Table 2.9 lists the loadings for the variables. A high loading (positive or negative) indicates a variable to be important to the principal component, whereas a low loading suggests the variable to be not important to that PC.

For the first PC the variables that appear not to be important are Mg yr1, Cu yr1, Cd yr2, Ni yr2, Ca yr3, Cd yr3, for the second PC the variables are: Cu yr1, a/b yr1, K yr2, Ca yr2, Ca yr3. Therefore Cu yr 1 and Ca yr 2 are not important to either PC. Chlorophyll 'a' and 'b for the year 1, year 2 and year 3 growth appear to be important for both PC's. Potassium and calcium are important in the year 1 and year 2 needles for the first PC only, and potassium has some importance in the year 3 growth. Calcium and potassium are both important plant nutrients [Mengel and Kirkby (1981)]. Nickel has some effect in the year 2 and year 3 needles for both principal components. Nickel may be important to plant growth but it is also known to be a plant toxin [Mengel and Kirkby (1981)].

Table 2.9. Loadings of the First and Second Principal Components for the 1986 Foliar Data.

Variable.	1st PC	2nd PC
Chl a yr1	0.2267	0.3098
Chl b yr1	0.2344	0.3230
a/b yr1	-0.1723	-0.1591
K yr1	0.2173	-0.1408
Ca yr1	0.2636	-0.1050
Mg yr1	-0.0342	0.3005
Cd yr1	0.1610	-0.1983
Ni yr1	0.2634	-0.1604
Cu yr1	0.0013	-0.0316
Chl a yr2	0.2634	0.1357
Chl b yr2	0.2980	0.1109
a/b yr2	-0.1780	0.0174
K yr2	0.2748	-0.0892
Ca yr2	0.2607	-0.0904
Mg yr2	-0.0586	0.1983
Cd yr2	-0.0909	-0.2023
Ni yr2	-0.0172	-0.1456
Cu yr2	0.1652	0.1373
Chl a yr3	0.1887	0.2122
Chl b yr3	0.1906	0.2391
a/b yr3	-0.1563	-0.2684
K yr3	0.1894	-0.2485
Ca yr3	0.0414	-0.0339
Mg yr3	-0.2280	0.2297
Cd yr3	0.0377	-0.1091
Ni yr3	0.1975	-0.2457
Cu yr3	0.2261	-0.2400

Fig. 2.4. Score Plot for the First PC versus the Second PC Showing the Site Distribution for the 1986 Foliar Data.

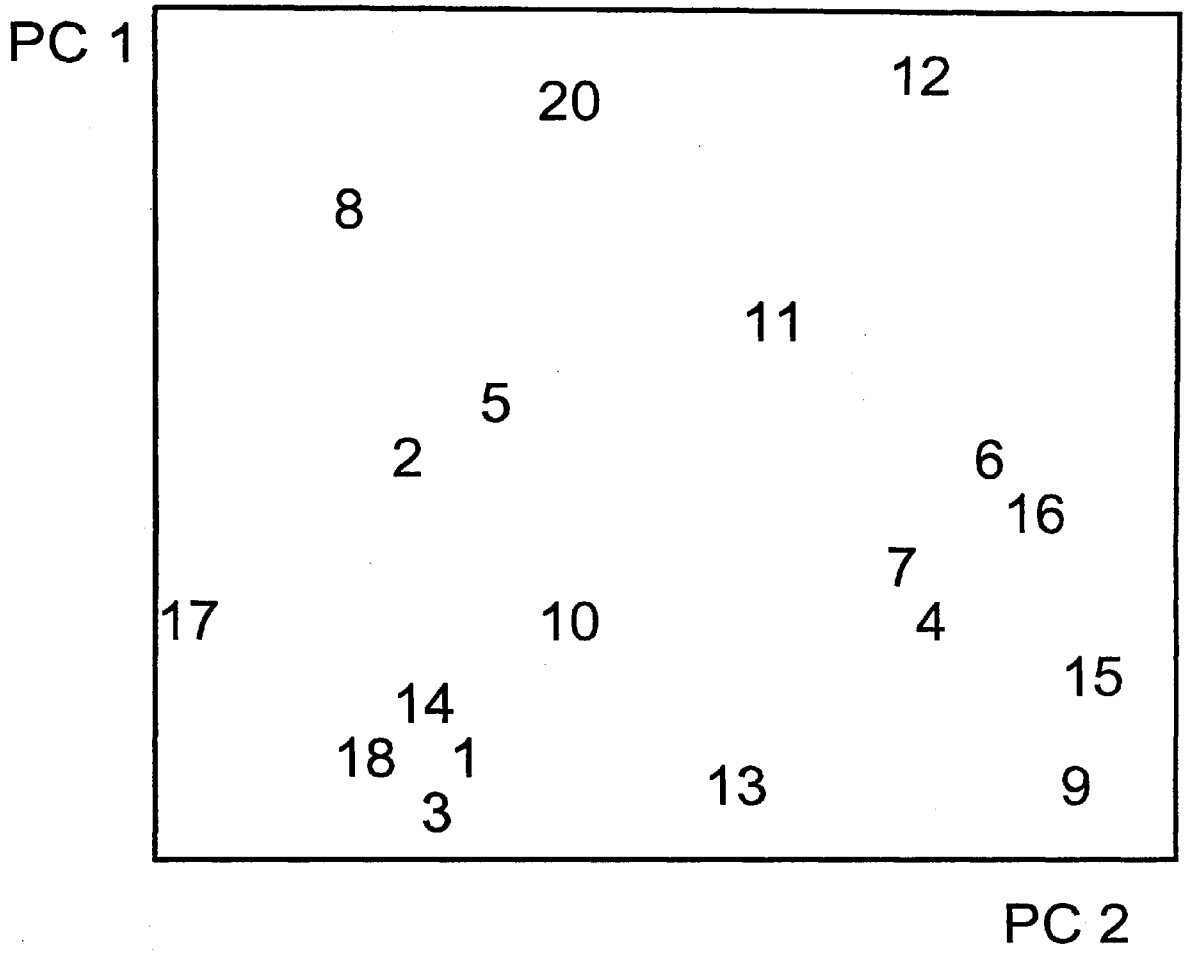
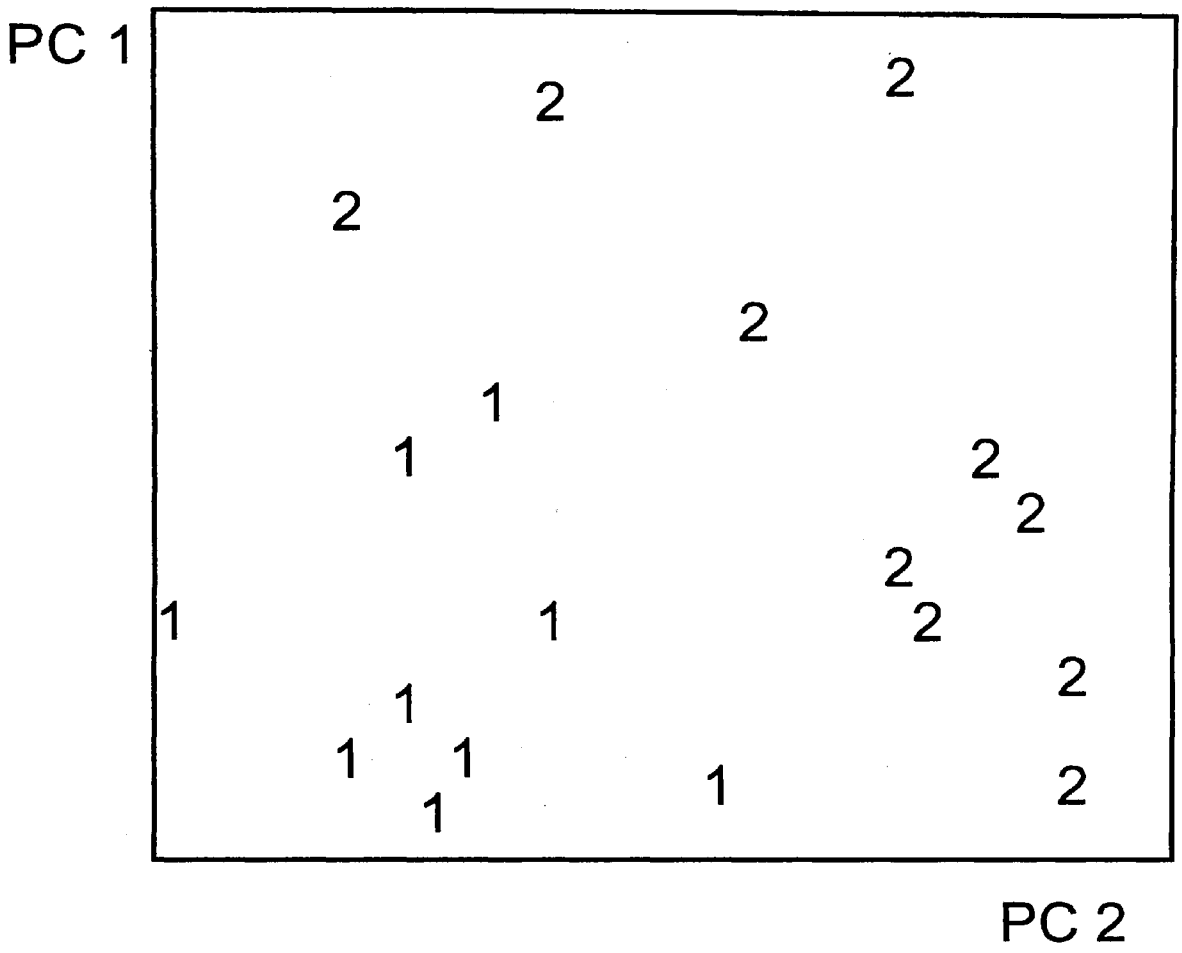


Fig. 2.5. Score Plot for First PC versus Second PC Showing the Class Distribution For the 1986 Foliar Data.



The PCA of the 1986 foliar data suggested the existence of two greatly distinct class types and therefore SIMCA (Soft Independent Modelling of Class Analogy) was used to determine if these classes exist and what variables are important in defining them. SIMCA can classify which object belongs to which particular class and it can give information as to which variables are important to each class.

The basic concept behind the SIMCA method is that multivariate data (in this case the foliar measurements) believed to form groups can be separated into different classes. Each class can be created by PCA, since a grouping of similar objects can usually be approximated by a PC-model, providing the measured variables express this similarity [Moseholm (1988)]. The dimensionality of the PC-model (A) is estimated to give the model the best predictive properties possible. As long as 'A' is less than one third of the number of observations the PC-model is well defined and stable. The residuals are calculated for each object in one class, which when properly added, determine a confidence interval around the model. By comparing this interval with 1) the distance to the objects belonging to other classes, 2) the ability of the model to fit the class, and 3) the significance of the class separations, the importance of the variables discriminating between classes can be determined [Moseholm (1988)]. SIMCA works best if each measurement is normally distributed in the measurement space. The modelling in a SIMCA analysis is scale dependent, thus the separate classes have to be scaled independently to unit variance and zero mean. The method lends itself quite well to outlier detection. They are easily identified since they usually lie well outside the class confidence intervals. The modelling power of a variable indicates how much it is participating in the model (in this case a division into two classes) [Wold (1981)]. A value of 0.2 or less indicates a variable to be irrelevant in the class and it may be deleted in further analyses [Wold (1981)]. Six components were extracted for both Class 1 and Class 2 models, and these best described the two class models.

Table 2.10. Modelling Powers of the variables for Both the Class 1 and Class 2

Variable	Class 1 Good Growth	Class 2 Poor Growth
Chl a yr 3	0.539	0.831
Chl b yr 3	0.520	0.849
a/b yr 3	0.759	0.406
K yr 3	0.611	0.605
Ca yr 3	0.190	0.452
Mg yr 3	0.723	0.873
Cd yr 3	0.361	0.916
Ni yr 3	0.526	0.087
Cu yr 3	0.221	0.345
Chl a yr 2	0.616	0.108
Chl b yr 2	0.477	0.331
a/b yr 2	0.374	0.700
K yr 2	0.261	0.245
Ca yr 2	0.667	0.174
Mg yr 2	0.598	0.900
Cd yr 2	0.516	0.534
Ni yr 2	0.494	0.090
Cu yr 2	0.626	0.575
Chl a yr 1	0.784	0.675
Chl b yr 1	0.391	0.684
a/b yr 1	0.256	0.499
K yr 1	0.677	0.521
Ca yr 1	0.868	0.633
Mg yr 1	0.249	0.508
Cd yr 1	0.414	0.000
Ni yr 1	0.482	0.888
Cu yr 1	0.997	0.339

From Table 2.10 it is seen that for the Class 1 model, Ca yr 3 does not appear to be contributing greatly. For the Class 2 model Cd yr 1, Ni yr2, Ca yr2 and Ni yr 3 do not appear to be contributing to it. Both year 3 chlorophylls are contributing to the two classes, as is the year 1 chlorophylls. For the year 2 needles, only the Class 1 chlorophyll 'a' is having a significant contribution to its class. Potassium is providing an important contribution to both classes for all three growth years, so is magnesium and to a lesser extent calcium. The other metals have some effect on the class structure as well. Cadmium is having no effect on the Class 2 sites for the year 1 since there were no detectable levels present in the samples.

A useful tool that helps to decide upon the existence of classes is the multiclass plot that is a representation of the object distances from each of the two classes. The object distances are calculated according to equation 1.

$$F_1 = S_p(1)^2 / S_o(1)^2 \qquad (1)$$

The test of significance is an F-test with $(M_1\text{-}A_1)$ and $(M_1\text{-}A_1)(N_1\text{-}A_1\text{-}1)$ degrees of freedom. Where M = Number of variables, N = number of class objects and A = number of principal components. This is the calculation used to decide whether an object belongs to a class.

S_o is the residual standard deviation (RSD), which is the typical measure of the distance between the class model and a sample belonging to the class and is worked out according to equation 2.

$$S_o^2 = \sum_{i=1}^{M} \sum_{k=1}^{M} \varepsilon_{ik}^2 / (M1 - A1)(N1 - A1 - 1) \qquad (2)$$

Sample P is classified as probably belonging to Class 1 if S_p (1) is not significantly larger than the typical class RSD. The Class 1 and Class 2 S_o and S_p values are given in Table 2.11. S_p is the residual standard deviation of the object and gives an estimation as to the degree of fit it has to the class model [Wold (1981)].

The MCL plot (Fig. 2.7) is quite clearly showing the existence of two distinct classes.

Fig 2.6. Multiclass Plot for the 1986 Foliar Data.

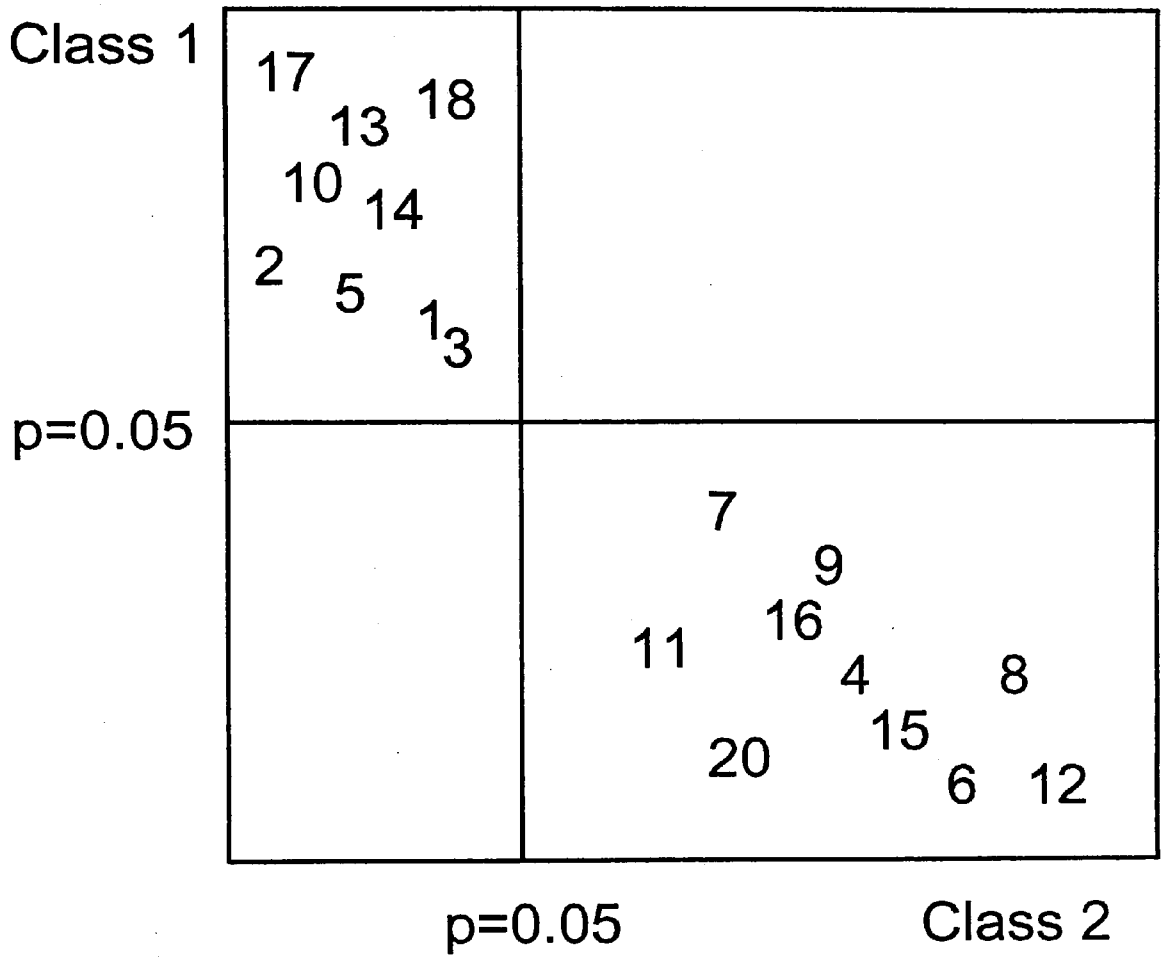


Table 2.11. Significance test for the Existence of Class 1 (Good growth) and the Class 2 (Poor growth) Sites at the Afan 1 Forest.

Site	Class 1 $S_o = 0.584$		Class 2 $S_o = 0.682$	
	S_p	F-value S_p^2 / S_o^2	S_p	F-value S_p^2 / S_o^2
1	0.136	0.054	1.622	5.661
2	0.216	0.137	1.460	4.583
3	0.196	0.113	1.594	5.469
4	2.019	11.961	0.334	0.239
5	0.316	0.293	1.114	2.668
6	2.014	11.904	0.167	0.060
7	1.529	6.856	0.692	1.031
8	2.408	17.000	0.186	0.073
9	1.837	9.897	0.581	0.727
10	0.112	0.037	1.527	5.027
11	1.276	4.774	0.390	0.326
12	2.729	21.844	0.097	0.021
13	0.446	0.583	1.078	2.501
14	0.272	0.217	1.281	3.529
15	1.979	11.491	0.238	0.121
16	1.873	10.295	0.371	0.296
17	0.156	0.071	1.751	6.597
18	0.410	0.494	1.525	5.005
20	1.644	7.932	0.221	0.105

The results given in Table 2.11 show that according to the F statistic for class 1 (F = 1.94), (good growth sites); 1, 2, 3, 5, 10, 13, 14, 17 and 18 belong to it since these values lie below the F cut-off value of 1.94. Similarly the sites that belong to Class 2 (poor growth) are; 4, 6, 7, 8, 9, 11, 12, 15, 16, 20. (F cut off value = 1.8). It was found that six PCs described the Class 1 data and Class 2 data after the PCs were estimated.

From the SIMCA, PCA and Cluster analysis of the foliar data evidently there are two site classes for the 1986 foliar data that compared well with the Forestry Commission growth data. Therefore it was decided to apply a Student's t-test to the individual parameters for

the good and poor growth foliar data. The significant results are shown in Table 2.12 (the rest of the results are given in Table 2, Appendix 2).

Table 2.12. Students t-test for the 1986 Foliar Data per Growth Year (mg kg^{-3})

YEAR 1 GROWTH

	CLASS 1 Good Growth		CLASS 2 Poor Growth		
	Mean	**sd**	**Mean**	**sd**	
K yr 1	5752.22	689.31	4960.40	544.38	**
Ca yr 1	3229.56	1541.26	1923.20	669.49	**
Cd yr 1	12.33	15.61	1.71	2.98	*
Ni yr 1	8.28	3.92	4.45	3.02	**

YEAR 2 GROWTH

	CLASS 1 Good Growth		CLASS 2 Poor Growth		
	Mean	**sd**	**Mean**	**sd**	
K yr 2	5328.7	777.4	3613.9	916.5	****
Ca yr 2	3946.1	2119.6	2226.6	624.3	**

YEAR 3 GROWTH

	CLASS 1 Good Growth		CLASS 2 Poor Growth		
	Mean	**sd**	**Mean**	**sd**	
K yr 3	4401.5	590.9	2866.7	513.7	****
Mg yr 3	501.0	83.1	815.0	282.1	***
Ni yr 3	7.7	1.3	4.58	1.3	****
Cu yr 3	4.5	0.9	3.29	0.7	***

Sig. Diff.		
	****	99.9%
	***	99.0%
	**	95.0%
	*	90.0%

In all three growth years, potassium was significantly higher in the Class 1 (good growth) trees. This is reasonable since potassium is essential for the well being of plants and the difference in the potassium levels may be one of the factors why the Class 2 (poor growth) trees are not doing as well as the Class 1 trees. It is also seen that potassium levels decrease from the younger year 1 tissues to the older year 3. This corresponds to the findings of Greenway and Pitman (1965) who found a similar trend in the younger parts of oat plants.

For the year 1 and year 2 growths, calcium is higher in the foliar tissues good growth trees. Calcium is also vital for the well being of plants [Mengel and Kirkby (1982)]. Calcium in the year 3 growth was found to be not significantly different in the two classes. Calcium has been linked to the synthesis of compounds found within plant cell walls and it has been linked with plant auxins [Burstrom (1968)]. Ozone exposure has been shown to cause modifications in plant membrane permeability that results in the ionic imbalance of potassium and calcium. This can upset many of the plants metabolic functions such as the regulation of stomatal response [Treshow and Anderson (1989)].

The three growth years were also subjected to a paired Student's t-test to see if there were any significant differences between them. Prior to this the differences between the year 1 and year 2, year 1 and year 3, year 2 and year 3 data were subjected to a Kolmogorov-Smirnov test for normality to see if a t-test could be applied. All the differences were found to be normally distributed except for Cd yr 3 - Cd yr 1 and Cd yr 2 - Cd yr 1 that were tested with the Wilcoxon signed non-parametric rank test. Table 2.13 shows the relevant data that was significantly different. The remainder of the results are given in Table 3 in Appendix 2.

Table 2.13 a. Paired Student's t-test for the Foliar Metals and the Chlorophylls for the Three Growth Years (mg kg^{-3}).

Variable	Mean	Diff.	sd	Prob.
Chl a yr 3	1256.58	203.52	470.20	*
Chl a yr 1	1053.06		319.36	
Chl a yr 1	1033.21	-308.84	306.95	***
Chl a yr 2	1342.05		417.23	
Chl b yr 3	456.59	123.83	216.66	**
Chl b yr 1	332.76		111.67	
Chl b yr 2	458.42	135.95	159.12	****
Chl b yr 1	322.47		109.70	
a/b yr 3	2.82	-0.46	0.28	****
a/b yr 1	3.28		0.37	
a/b yr 2	3.01	-0.32	0.51	**
a/b yr 1	3.33		0.37	
K yr 3	3588.94	-1730.65	953.03	****
K yr 1	5319.59		743.17	
K yr 3	3588.94	-864.12	953.03	****
K yr 2	4453.06		1279.74	
K yr 2	4426.16	-909.31	1209.30	***
K yr 1	5335.47		724.12	
Ca yr 2	3041.11	499.11	1723.28	***
Ca yr 1	2542.00		1314.91	
Mg yr 3	667.24	-417.76	262.52	****
Mg yr 1	1085.00		206.96	
Mg yr 1	1058.21	317.74	215.65	****
Mg yr 2	740.47		195.02	
Ni yr 1	6.27	2.49	3.90	**
Ni yr 2	3.78		2.20	
Ni yr 2	3.98	-2.08	2.12	***
Ni yr 3	6.06		2.04	
Cu yr 1	2.45	-1.43	1.59	***
Cu yr 3	3.88		0.99	
Cu yr 2	2.31	-1.57	0.94	****
Cu yr 3	3.88		0.99	

Table 2.13b. Wilcoxon Signed Rank Test For Cd yr 1 and yr 2 Needles

Cd yr 2	Z = -2.4303	**
Cd yr 1		

Sig. Diff.	****	99.9%
	***	99.0%
	**	95.0%
	*	90.0%

The results of the paired t-test show that the potassium levels are highest in the newest growth i.e. the year 1 growth. Potassium levels increase from the oldest to the youngest. This again supports the findings of Greenway and Pitman (1965), who found potassium levels to be the highest in the younger parts of the plant. Only calcium between the years 1 and 2 showed any significant difference.

The highest chlorophyll a and b levels were found in the year 2 growth, with both the year 2 and year 3 needles having higher levels than the youngest needles. The work carried out on the pigment levels of Norway spruce by Blintsov and Asyutin (1983) also found the year 1 needles to have lower levels of pigments than the year 2 and year 3 needles.

The highest chlorophyll 'a' to chlorophyll 'b' (a/b) ratio was found to be in the year 1 growth. The a/b ratio of all the sites was approximately 3:1 as also found by Lichtenthaler (1987). This ratio can be modified by growth conditions and environmental factors. High light and sun exposed plants can show a/b ratios between 3.2 and 4. Conversely, plants growing in shady conditions can exhibit a/b ratios of 2.5 to 2.9 [Seybold and Egle (1970)].

Nickel was found to be significantly higher in the year 1 needles compared with the year 2 needles and significantly higher in the year 3 needles compared with the year 2. Nickel in excessive quantities is toxic to plant life [Morgan (1983)]. The nickel concentration in plants growing on soils other than those with high levels of nickel are usually < 10 ppm [Hutchinson (1981)]. The highest level of nickel in the Sitka spruce at the Afan 1 site is 6.3 ppm. This may be high enough to cause damage as the upper critical tissue concentration of Ni has been shown to be 5.8 ppm for Sitka spruce seedlings [Morgan (1983)]. The upper critical concentration of an element in a plant is said to occur when

the level of the element causes a decrease in the yield of the plant [Beckett and Davis (1977), Burton et al (1986)].

Copper concentrations were found to be significantly different between the year 1 and year 3 growth and the year 2 and year 3 growth, with the year 3 growth having the higher copper levels. Copper may exhibit a variable mobility within plants, the rate and pattern of the mobility being controlled by internal and external factors [Lepp (1981)]. Copper is an essential element to plant growth. Many enzyme systems require copper as a co-factor [Phipps (1976)]. Copper deficiency leads to a reduction in the lignification and thickening of cell walls and xylem elements [Lepp (1981)].

Copper toxicity can arise due to enrichment of the soil, due to the presence of natural copper mineralisation [Lepp (1981)]. Excess Cu interferes with the normal metabolic functions of the plant such as blocking specific enzyme reactions that results in the cessation of growth [Treshow (1970), Foy et al (1978), Ormrod (1984)]. The upper critical tissue concentrations for copper have been shown to be 88 ppm for Sitka spruce seedlings [Morgan (1983)]. The Cu levels of the trees at the Afan 1 sites are below this.

Cadmium concentrations in the needles were found to be significantly higher in the year 1 growth as compared to the year 2. Cadmium is known to be a very toxic element for plants and living organisms in general [Lepp (1981), Morgan (1983)]. Atmospheric sources are principally from fertilisers, industrial processes containing zinc, pesticides and metal processing [Treshow and Anderson (1989)]. Toxicity in plants due to cadmium first results in reduced photosynthetic and transpiration rates, followed by general chlorosis [Ormrod (1984)]. Cadmium has also been shown to affect plant root growth [Malone et al. (1978)]. Normal background cadmium levels are generally under the 1 ppm level on a dry weight basis [Page et al. (1981)]. The levels at the Afan site for

foliar cadmium are generally below 1 ppm, though some of the year 1 growth exhibited possibly abnormally high levels of cadmium: these are site 3 (30.21 ppm), site 10 (27.8 ppm) and site 17 (38.4 ppm), and may be the result of particulate matter lodged in the stomata. On the whole the cadmium levels may be considered below the background level. Morgan (1983) suggests the upper critical tissue concentration of Cd in Sitka spruce seedlings to be 4.8 ppm. Thus there may be some Cd toxicity at the Afan sites.

2.8 Statistical Analysis of the Soil Solution Elemental Analysis.

2.8.1. PCA of the Soil Solution Elements.

The data for the soil solution analysis were given by the Forestry Commission. The sampling was carried out in January 1986. The score plots (Fig. 2.8 and Fig. 2.9) indicate the possible existence of three groups. These groups can be split as follows: the first group consisting of sites 1, 6, 15 and 16; the second (and largest) group consisting of sites 2, 3, 5, 7, 8, 10, 11, 12, 13, 14 and 17; and the third group containing sites 9 and 20. Site 4 appears to be an outlier. There is not the clear split of the data into the classes that the foliar analysis provided.

Fig. 2.7. Score Plot for First PC versus Second PC Showing the Site Distribution For the 1986 Soil solution Data.

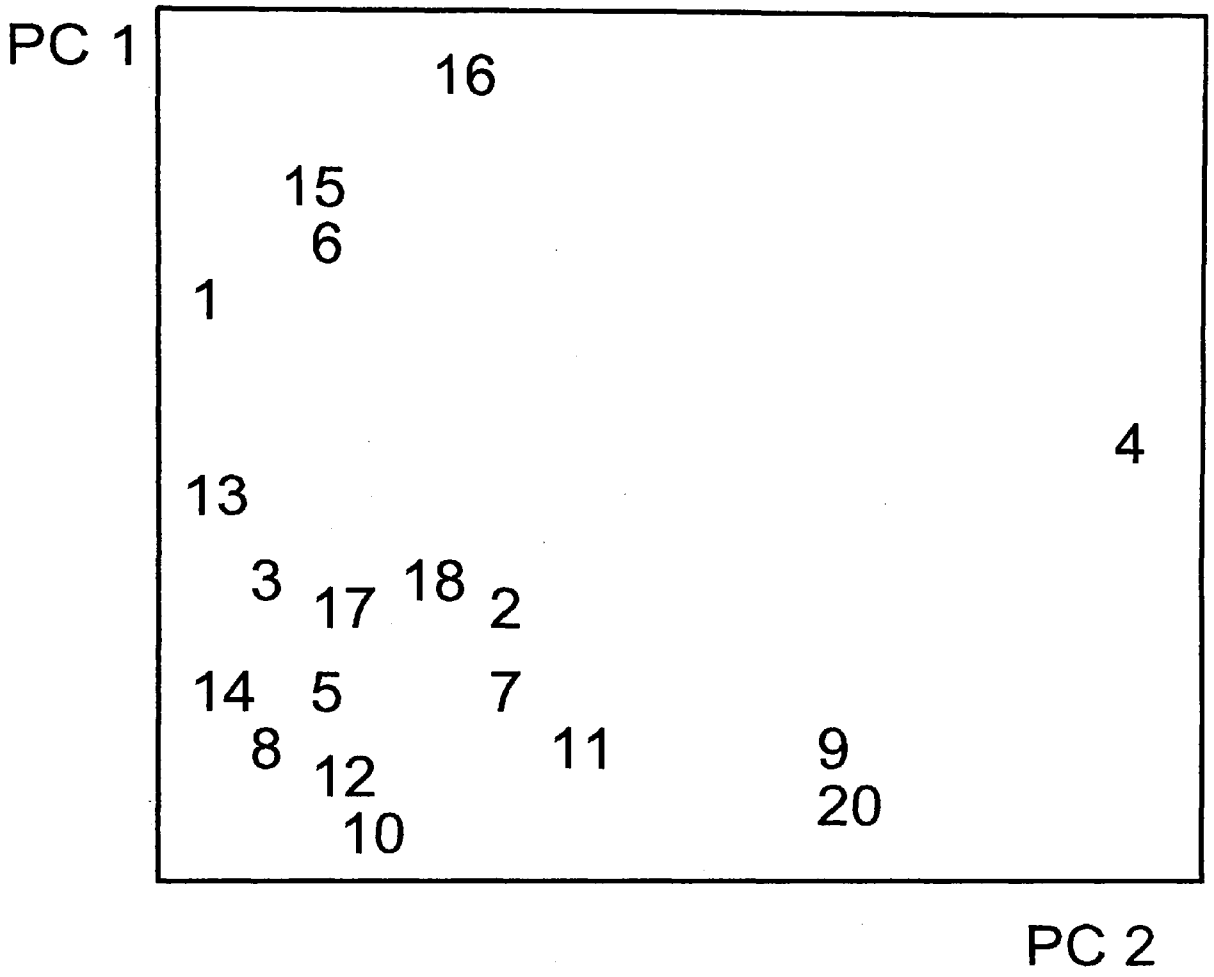
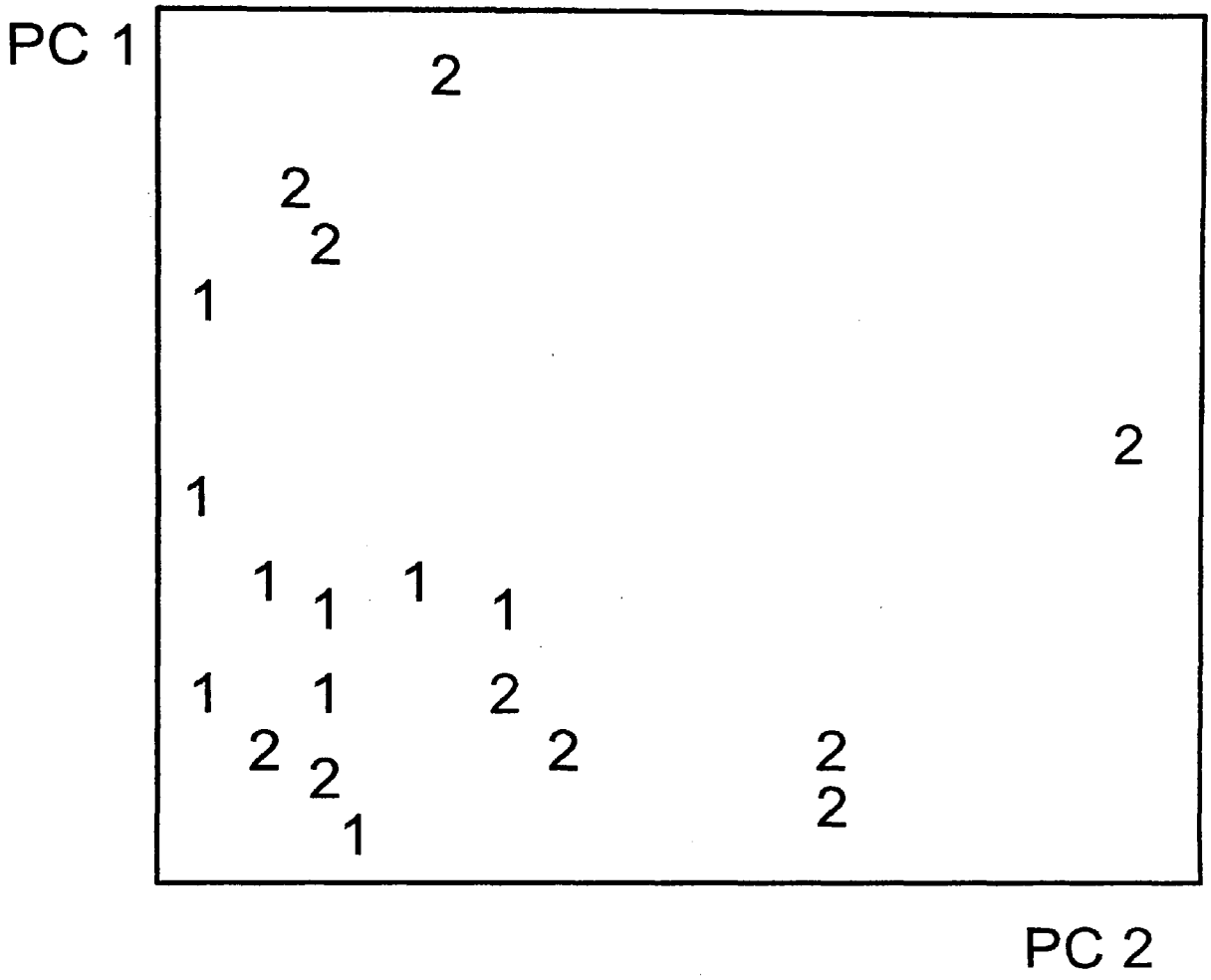


Fig. 2.8. Score Plot for First PC versus Second PC Showing the Class Distribution For the 1986 Soil Solution Data.



2.8.2 SIMCA Analysis of the Soil Solution Data

The SIMCA package was used to determine whether the soil solution data could be classified into the good and poor growth categories as decided from the 1986 growth parameter data analysis. Six PC's for Class 1 and 4 PC's for Class 2 appeared to give the best model after estimation of the PCs. The modelling powers for the variables are given in Table 2.14.

Table 2.14. Modelling power for the Soil Solution Elemental Variables.

Variable	Class 1 Good Growth	Class 2 Poor Growth
pH	0.999	0.554
Al	1.000	0.503
As	1.000	0.229
B	1.000	0.760
Ca	1.000	0.359
Cd	0.000	0.705
Cu	1.000	0.000
Fe	1.000	0.780
K	1.000	0.548
Mg	1.000	0.538
Mn	1.000	0.771
Na	1.000	0.674
Ni	1.000	0.469
P	1.000	0.784
S	1.000	0.531
Si	1.000	0.642
Zn	1.000	0.642

The modelling power shows that some elements are not contributing to the two class models, these are namely, Cd for Class 1 and Cu for Class 2. Cadmium was below detectable limits for the Class 1 sites and thus would have no contribution at all. The multiclass plot (Fig. 2.10) shows the Class 2 envelope encloses the Class 1 envelope except for site 1. Effectively this means that it is not possible to classify the sites on the basis of the soil solution elemental analysis. The F- values for the sites and hence their classifications are given in Table 2.15.

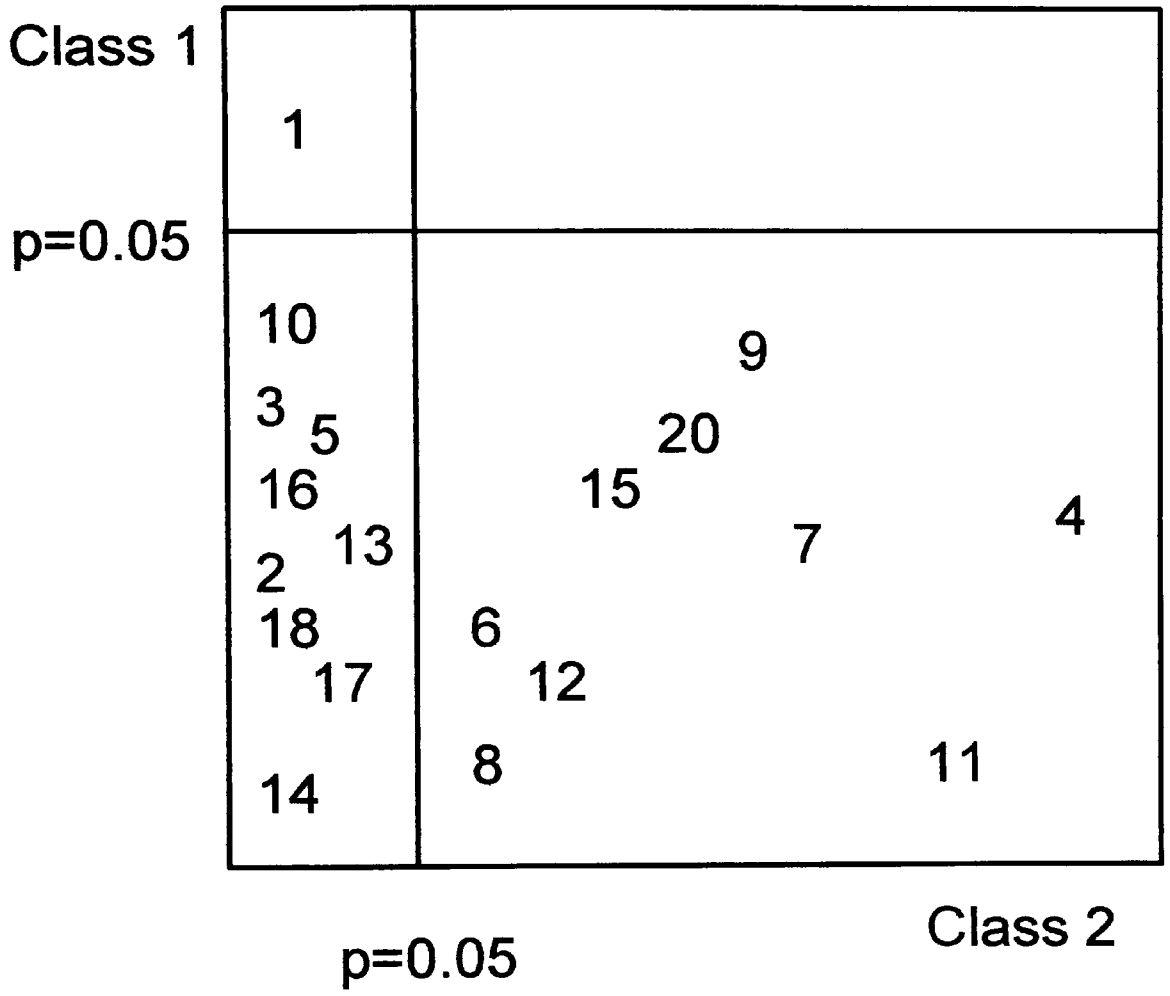
Table 2.15. Significance Test for the Class 1 (good) and Class 2 (poor) Soil Data.

	Class 1 S_0 =4.623E-04		Class 2 S_0 = 0.569	
Site	**S_n**	**F-value S_n^2/S_0^2**	**S_n**	**F-value S_n^2/S_0^2**
1	1.169E-04	0.069	1.159	4.149
2	1.156E-04	0.067	0.592	1.083
3	1.156E-04	0.067	0.547	0.924
4	11.560	6.71E10	0.300	0.278
5	1.155E-04	0.070	0.365	0.411
6	1.486	1.109E7	0.379	0.444
7	5.502	1.52E8	0.380	0.447
8	1.291	8.37E6	0.163	0.082
9	4.808	1.16E8	0.613	1.161
10	1.148E-04	0.066	0.759	1.780
11	8.582	3.69E8	0.089	0.024
12	2.450	3.01E7	0.344	0.043
13	1.158E-04	0.067	0.313	0.303
14	1.1566E-04	0.067	0.221	0.151
15	2.642	3.51E7	0.537	0.891
16	1.697	1.45E7	0.326	0.329
17	4.041E-04	0.820	0.307	0.294
18	4.041E-04	0.820	0.307	0.294
20	3.467	6.04E7	0.562	0.978

Class 1 number of degrees of freedom are 10/20

Class 2 number of degrees of freedom are 12/36

Fig 2.9 Multiclass Plot for the Soil Solution Elemental Data.



2.8.3. Student's t-test Analysis of the Soil Solution Data.

Even though the multivariate analyses performed on the soil solution elemental data revealed no relevant classes, the data was kept to the same classification of the sites as determined by SIMCA for the 1986 foliar results. This was done in order to determine if there were any significant differences in the soil solution elemental levels that could indicate possible causes as to why there are the significant differences in the tree growth classes. The remainder of the results are given in Appendix 2.

Table 2.16. Student's t-test for the Soil solution Data.

	Class 1 Good Growth		Class 2 Poor Growth		
	Mean	**sd**	**Mean**	**sd**	
Cd	0.00	0.00	0.007	0.01	**
Mn	0.03	0.03	0.140	0.25	**
Na	7.60	1.36	11.000	3.94	**
Zn	0.60	0.16	1.000	0.37	**
B	0.26	0.07	0.420	0.22	*
Ca	3.80	3.12	4.180	2.19	*
Cu	0.03	0.01	0.050	0.02	*
S	4.10	2.22	6.800	3.19	*

Sig. Diff. ** 95.0%
* 90.0%

The soil solution Ca levels were found to be significantly higher at the poor growth sites (90% significance). It was found that the good growth sites had significantly higher levels of Ca in the year 1 and year 2 needles. As discussed in the introduction, it is quite difficult to get any meaningful results from soil elemental analysis, due to the innate variability of soils. The CEGB only examined the soil solution for metals. This does give any indication to the total concentrations or any idea of the levels of exchangeable cations that might be present.

2.8.4 Comparison of Sites with respect to Elevation.

One interesting point to come out from this survey was revealed by performing a Student's t-test on the elevations of the 19 sites used in this survey. The elevations of the sites are given in Table 2.17 and the result of the t-test is given in Table 2.18.

Table 2.17. Elevations of the 19 Sites at the Afan 1 Forest.

Site No	Elevation (metres)
1	470
2	460
3	440
4	515
5	500
6	500
7	500
8	510
9	520
10	390
11	480
12	500
13	490
14	460
15	515
16	525
17	510
18	475
20	460

Table 2.18. Student's t-test Result for the Elevations of the 19 Afan 1 Forest Sites

	No. of Cases	Mean	sd	se	F value	2-tailed Prob.
Class 2	10	502.5	19.76	6.25		
					3.30	0.095
Class 1	9	466.11	35.86	11.95		

Pooled Variance Estimate			Separate Variance Estimate		
t-value	df	2-tailed Prob.	t-value	df	2-tailed Prob
2.78	17	0.013	2.70	12.16	0.019

The result of the t-test shows that there is a significant difference (> 95%) between the elevations of the two classes of sites at the Afan 1 Forest, with the poor growth sites being at the higher elevation. This could be an important factor in going some way to explaining why there are marked differences in growth exhibited by these sites. The sites at the higher elevations may be experiencing a greater amount of leaching of nutrients from their respective soils resulting in poor growth though the Ca levels in the soil solution were significantly higher for the poor growth sites as compared to the good growth sites. Other nutrients essential to tree growth may in fact be leached. Another possible factor is that the trees at the higher elevations may be more exposed, which may result in wind damage. Also acid mists may be more prevalent at the higher elevations leading to leaching of nutrients from the needles; ozone and other photochemical pollutants may also be more prevalent at the higher altitudes. [Cape and Unsworth (1988), Chevone and Linzon (1988), Treshow and Anderson (1989)] Decreased growth at higher elevations has been linked to air pollution [Prinz et al (1982), Cape et al (1988)]. The effects of ozone and acid mists have been shown to have a greater effect upon trees at higher elevations [Cape and Unsworth (1988), Davison et al (1988), Chevone and Linzon (1988)]. This may be the cause of the decreased growth observed at the poor growth sites at the Afan 1 Forest.

A comparison of the loss of potassium from the three needle ages obtained from the poor growth and good growth sites yielded some interesting observations. Using the year 1 needles as 100% it appears that the good growth sites show a lower potassium loss as compared to the poor growth sites (Table 2.19). This loss may be indicative of increased leaching of potassium from the trees at the poor growth sites that occur at the higher elevations. The leaching may be due to a combination of ozone, acid rain and acid mist that can damage the waxy cuticle [Percey et al (1990)] and the needles [Davison et al (1988)]. This can lead to leaching out of nutrients such as potassium, calcium and magnesium This has been shown to occur in fumigation experiments on spruce seedlings where they were exposed to combinations of ozone and sulphur dioxide with a background acid rain [Prinz et al (1987), Wellburn (1988)]. This was found to cause leaching out of the needles of calcium and magnesium . The lower potassium levels in the poor growth trees may also be as a result of mechanical injury due to wind again leading to increased leaching of nutrients [Treshow and Anderson (1989)].

Table 2.19. Comparison of Potassium Levels for the Needles of the Three Growth Years for the Good and Poor Growth Sites at the Afan 1 Forest.

	Good Growth		Poor Growth		
	K mg/kg	% diff.	K mg/kg	% diff.	Sig. diff. Good/Poor
Year 1	5752	0.0	4960	0.0	**
Year 2	5328	-7.4	3613	-27.1	****
Year 3	4401	-23.1	2866	-42.2	****

CHAPTER 3

3. EPICORMIC TISSUE ANALYSIS.

All trees produce epicormic shoots from the main stem or branches. They originate from suppressed buds embedded in the bark [Patch et al (1986), Wignall et al (1987)]. However, it has been observed that trees in decline produce a greater number of these epicormic shoots [Coutts (Pers. Comm.) (1987)]. The loss of apical dominance is a clear characteristic of trees exhibiting epicormic growth. Initially the epicormic tissues appear to be healthy and can form a replacement crown. However, after a few years the epicormic shoots themselves go into decline [Coutts (Pers. Comm.) (1987)].

It is not clear why epicormic shoots are produced but it may be as a result of defoliation [Rose (1985)] and the defoliation may be due to pollution or mechanical damage caused by the natural elements. It is not known whether there are any differences in chlorophyll and metal concentrations between the epicormic and the normal tissues and this section of work was carried out to determine this.

3.1 Foliar Sampling and Results.

The foliar tissues were sampled in late October and early November 1987. The branches were removed from the third whorl down from the apical shoot. The chlorophyll and metal concentrations were determined according to the method described in chapter 2, with the addition that zinc was included in the foliar analysis. The results are given in Table 3.1 for the year 1 normal tissues and Table 3.2 for the year 1 epicormic tissues.

Table 3.1 Chlorophyll Levels (mg kg^{-1} fresh weight) and Metal Concentrations for the Normal Tissues (mg kg^{-1} dry weight).

Site	Chl a	Chl b	a/b	K	Ca	Mg	Cd	Ni	Cu	Zn
1	570	161	3.54	4016	2641	1247	0.0	7.8	6.0	38.2
2	803	231	3.54	4776	2894	1361	2.0	6.0	2.0	45.9
3	775	225	3.44	4519	1259	1097	3.0	5.0	9.0	27.2
4	616	167	3.48	3130	959	1469	0.0	4.8	4.0	23.9
5	776	204	3.80	3350	2486	1086	0.0	2.0	5.0	20.9
6	500	127	3.94	2671	1197	1431	3.0	1.9	4.0	56.4
7	669	198	3.38	2863	1858	1250	1.0	6.0	9.0	59.8
8	1023	328	3.12	5858	3010	946	0.0	6.0	4.0	50.3
9	499	162	3.08	4948	1510	1210	0.0	3.0	3.0	29.0
10	843	246	3.43	5475	2155	1733	0.0	11.2	4.0	28.5
11	737	199	3.70	3492	2740	1357	1.0	12.0	5.0	20.4
12	653	190	3.70	6121	1838	1671	0.0	3.9	6.0	32.8
13	681	226	3.01	5187	1823	1470	0.0	8.0	4.0	8.4
14	882	261	3.38	4396	1122	779	2.0	8.0	2.0	57.2
15	608	167	3.64	2624	1091	1574	1.0	5.9	8.0	29.5
16	558	161	3.47	5237	1110	1386	0.0	4.9	3.0	9.4
17	757	217	3.49	4048	1818	1388	0.0	2.0	5.0	29.8
18	903	295	3.06	7301	2386	1193	0.0	6.0	4.0	2.1
20	643	190	3.38	4762	1883	1567	0.0	9.9	3.0	27.1

0.00 = below detectable limit

Table 3.2 Chlorophyll Levels (mg kg^{-1} fresh weight) and Metal Concentrations for the Epicormic Tissues (mg kg^{-1} dry weight).

Site	Chl a	Chl b	a/b	K	Ca	Mg	Cd	Ni	Cu	Zn
1	827	256	3.23	3130	2337	1253	3.0	3.0	3.0	78.2
2	1365	439	3.11	1983	6032	917	0.0	0.0	4.0	62.1
3	910	302	3.01	2005	3871	1602	0.0	0.0	2.0	37.5
4	660	182	3.63	2875	2260	2052	2.0	4.0	14.0	50.2
5	1077	333	3.23	7094	1122	1228	1.0	5.8	2.0	50.2
6	478	121	3.95	2118	1270	1155	2.0	4.0	0.0	22.2
7	701	206	3.40	2371	1249	1256	0.0	1.0	4.0	48.2
8	808	248	3.26	6310	3010	1178	0.0	4.0	5.0	55.6
9	484	153	3.16	4329	1636	1222	0.0	2.0	3.0	52.1
10	798	255	3.13	8943	2260	1924	0.0	13.3	6.0	62.6
11	747	204	3.66	4102	1545	1322	0.0	5.9	4.0	21.9
12	597	202	2.96	8804	2155	1824	0.0	7.8	5.0	52.7
13	644	208	3.10	7515	2034	1501	0.0	5.8	4.0	16.9
14	895	258	3.47	3763	2071	1336	6.0	14.0	4.0	30.2
15	590	144	4.10	2942	1749	928	3.0	4.2	0.0	37.0
16	781	240	3.25	5034	1674	1189	0.0	3.8	2.0	11.3
17	792	251	3.16	3916	2790	1485	0.0	0.0	1.0	24.8
18	913	302	3.02	7403	2885	1534	0.0	6.0	6.0	37.5
20	675	201	3.36	4320	1496	1621	0.0	4.7	2.0	8.2

0.00 = below detectable limit

3.2 Univariate Analysis of Tissues.

If epicormic tissues are produced as a result of stresses, then the chlorophyll levels and the concentrations of the trace metals may be affected. Potential differences between the individual nutrients and the chlorophyll levels were investigated using a paired Student's t-test (Table 3.4). All the results were found to be normally distributed (Table 3.3).

Apparently from the result, the epicormic and the normal tissues are not substantially different on the basis of the individual variables. This is surprising as visually, a tree possessing a large degree of epicormic growth appears "greener". This may be due to there being a greater degree of epicormic growth as compared to the normal foliage. The normal foliage on a tree under stress is less healthy in appearance as compared to the epicormic tissues.

Table 3.3. Kolmogorov-Smirnov Test for Normality in Nutrients and Chlorophyll Levels on Differences Between Normal Tissues and Epicormic Tissues.

			Most Extreme Differences				
Variable	**difference mean**	**sd**	**absolute**	**positive**	**negative**	**K-S Z**	**2-tail Prob.**
Chl a	-65.58	169.66	0.29	0.18	-0.29	1.253	0.087
Chl b	-16.84	48.40	0.25	0.15	-0.25	1.099	0.179
a/b-ratio	0.08	0.25	0.22	0.22	-0.14	0.967	0.307
K	-328.00	1605.63	0.22	0.17	-0.22	0.958	0.318
Ca	-192.95	927. 22	0.10	0.10	-0.10	0.444	0.989
Mg	-121.68	310.03	0.15	0.15	-0.10	0.648	0.795
Cd	-0.42	1.54	0.29	0.23	-0.29	1.273	0.078
Ni	1.32	3.60	0.15	0.09	-0.15	0.650	0.792
Cu	0.95	4.06	0.13	0.13	-0.13	0.561	0.911
Zn	-7.53	21.25	0.09	0.09	-0.09	0.384	0.998

Table 3.4. Student's Paired t-test for the Epicormic and Normal Tissues E = epicormic

Variable	No. of Cases		Mean		s.d		se
Chl a	19		710.32		142.10		32.60
E Chl a	19		775.90		208.31		47.79
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-65.58	169.58	38.92	0.588	0.008	-1.68	18	0.109
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Chl b	19		199.21		59.80		13.72
E Chl b	19		216.05		69.76		16.00
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-16.84	48.41	11.11	0.731	0.000	-1.52	18	0.147
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
a/b	19		3.30		0.72		0.16
E a/b	19		3.22		0.81		0.19
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
0.08	0.25	0.06	0.954	0.000	1.46	18	0.162
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
K	19		4344.53		1465.58		336.23
E K	19		4672.53		2362.59		542.02
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-328.00	1605.63	368.36	0.744	0.000	-0.89	18	0.385
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Ca	19		1777.89		654.27		150.10
E Ca	19		1970.84		839.79		192.66
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-192.95	927.22	212.72	0.249	0.304	-0.91	18	0.376
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Mg	19		1274.47		328.50		75.36
E Mg	19		1396.16		309.83		71.08
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-121.68	310.03	71.13	0.530	0.020	-1.71	18	0.104
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Cd	19		0.58		1.02		0.23
E Cd	19		1.00		1.63		0.38
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-0.40	0.09	0.35	0.401	0.089	-1.19	18	0.249

Table 3.4 (Contd.)

Variable	No. of Cases		Mean		s.d		se
Ni	19		6.01		2.94		0.67
E Ni	19		4.70		3.86		0.89
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
1.32	3.60	0.83	0.466	0.044	1.59	18	0.129
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Cu	19		4.68		2.11		0.48
E Cu	19		3.74		3.05		0.70
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
0.95	4.06	0.93	-0.212	0.383	1.02	18	0.323
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Zn	19		29.20		17.08		3.92
E Zn	19		36.73		20.20		4.64
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-7.53	21.25	4.88	0.360	0.130	-1.54	18	0.140

3.3 Multivariate Analysis of the Epicormic and the Normal Tissues.

Principal Component Analysis was performed on the data to see if there were any groupings of the sites. The score plots for the epicormic and the non-epicormic shoots (Fig. 3.1 and Fig. 3.2) show that there is some agreement between the groupings here and those determined for the 1986 foliar data. Again site 8 appears to fall between the good and poor growth classes.

Fig 3.1 Score Plot for the First PC versus the Second PC Showing the Site Distribution.

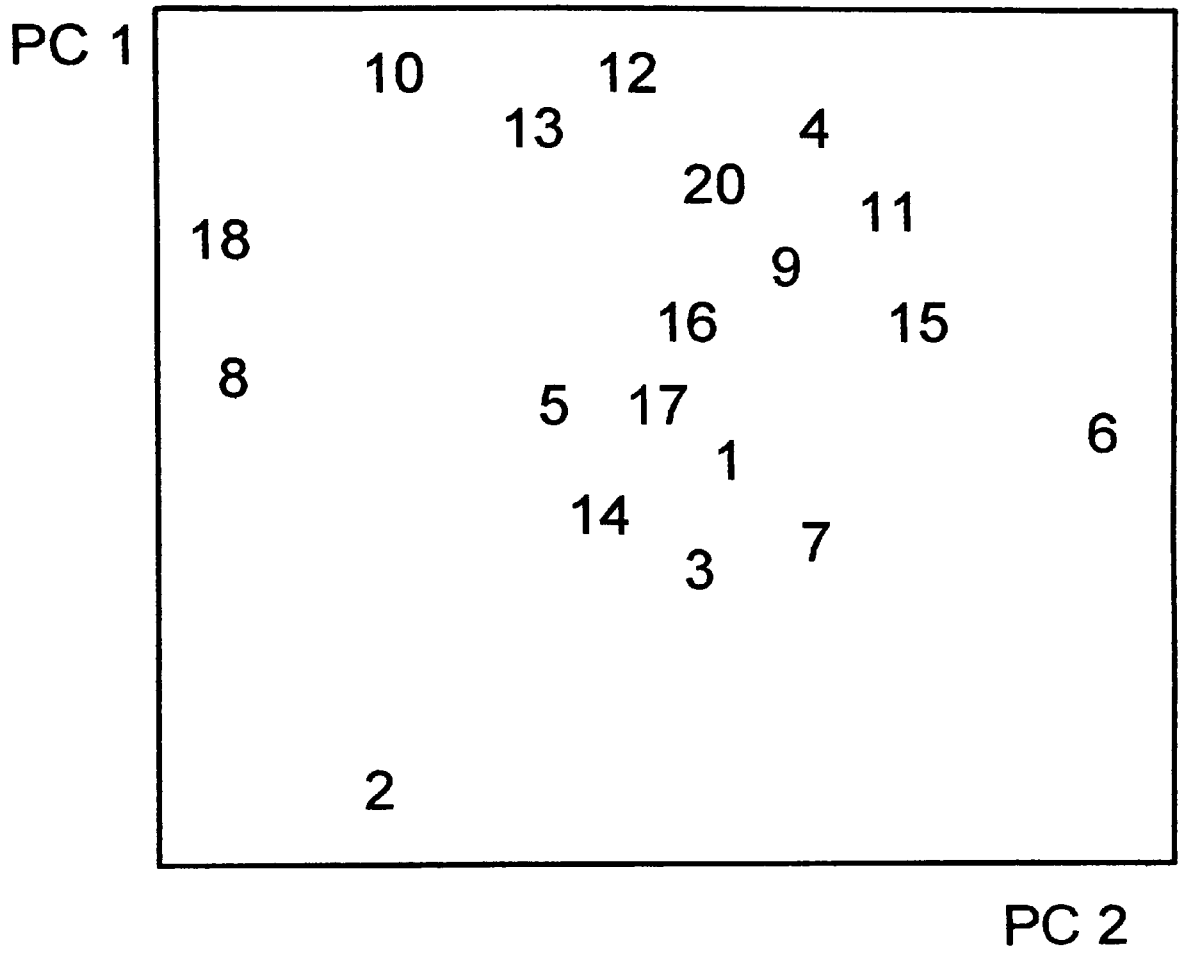
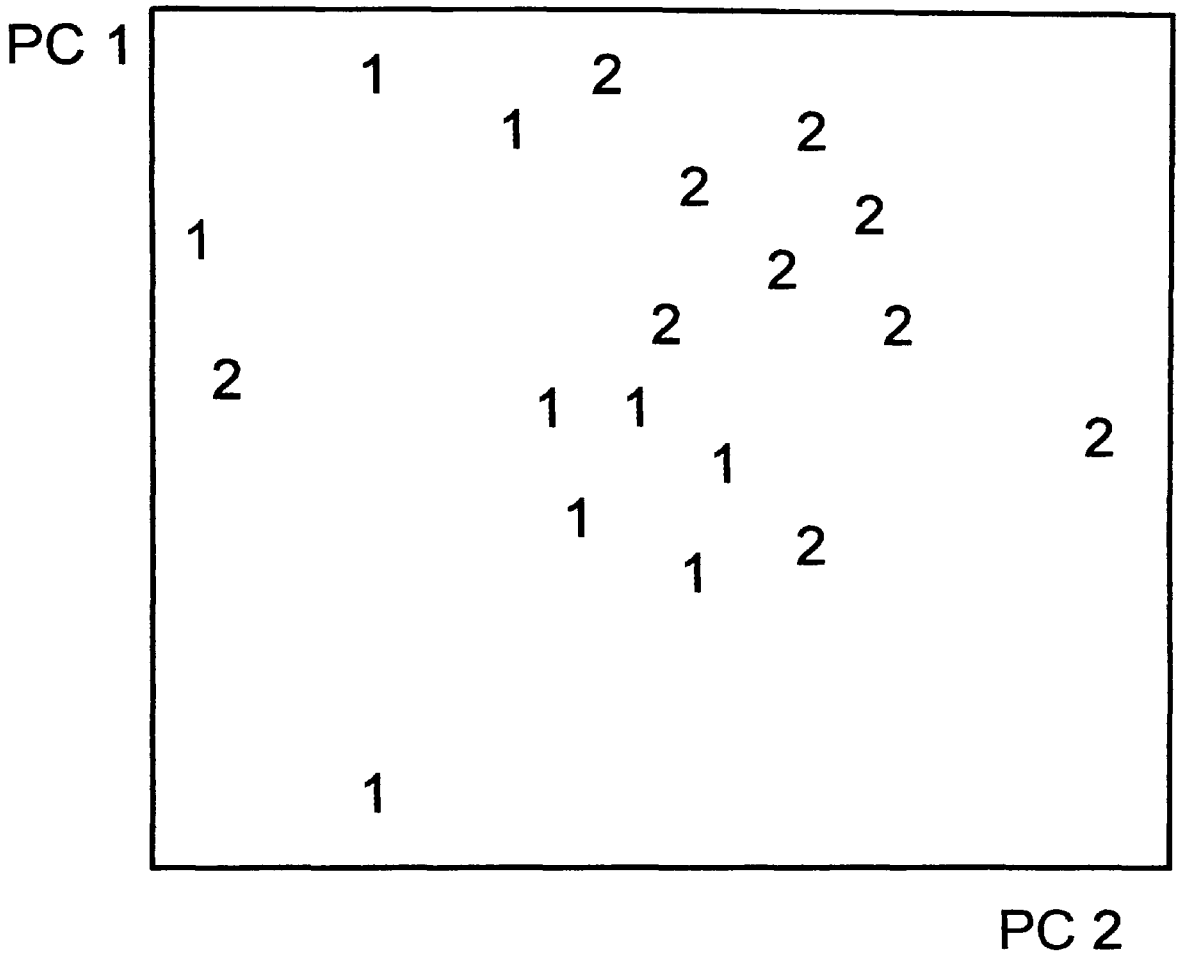


Fig 3.2 Score Plot for the First PC versus the Second PC Showing the Class Distribution.



To verify this result SIMCA was used to determine the class structures. The classes were set up as indicated by the PCA i.e. Class 1 consisting of sites 1, 2, 3, 5, 8, 10, 13, 14, 17 and 18 and Class 2 consisting of sites 4, 6, 7, 9, 11, 12, 15, 16 and 20. This is slightly different from that obtained from the 1986 data, as site 8 appears to be a Class 1 site. Table 3.5 gives the information dealing with the structure of both classes.

Table 3.5. Significance Test for the Class 1 (good) and Class 2 (poor)

Site	Class 1 $S_0 = 0.526$		Class 2 $S_0 = 0.638$	
	S_p	S_p^2/S_0^2	S_p	S_p^2/S_0^2
1	0.307	0.342	0.978	2.377
2	0.176	0.111	2.720	18.163
3	0.179	0.116	1.618	6.427
4	1.971	14.027	0.149	0.055
5	0.170	0.104	1.417	4.929
6	1.481	7.921	0.285	0.120
7	0.717	1.857	0.427	0.449
8	1.043	3.933	0.169	0.070
9	0.799	2.306	0.281	0.194
10	0.165	0.098	0.889	1.940
11	1.138	4.679	0.376	0.348
12	1.191	5.124	0.224	0.124
13	0.266	0.255	0.530	0.689
14	0.030	0.003	2.010	4.933
15	0.851	2.618	0.511	0.641
16	1.737	10.904	0.467	0.535
17	0.410	0.608	0.931	2.129
18	0.317	0.362	0.773	1.467
20	0.752	2.041	0.396	0.385

The modelling power for the two classes derived from the SIMCA analysis is given in Table 3.6. Again the modelling power gives an indication as to the significance of variables in the two classes.

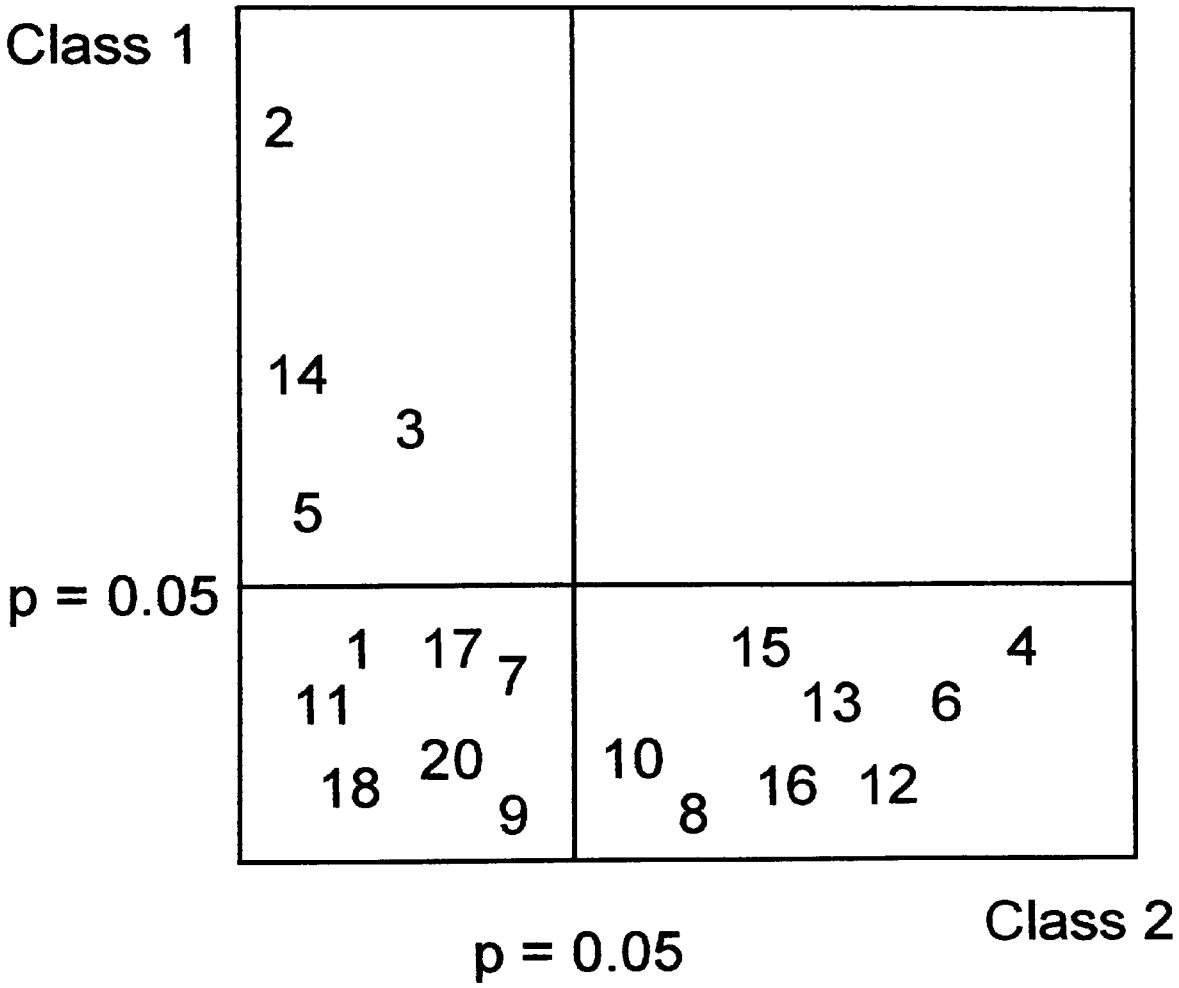
Table 3.6. Modelling Power for the Different Variables in the 1987 Foliar Data

Normal - Year 1		
Variable	**Class 1 M.P**	**Class 2 M.P.**
Chl a	0.68	0.69
Chl b	0.65	0.76
a/b	0.86	0.80
K	0.47	0.64
Ca	0.68	0.31
Mg	0.09	0.63
Cd	0.57	0.61
Ni	0.72	0.64
Cu	0.34	0.12
Zn	0.41	0.25
Epicormic - Year 1		
Variable	**Class 1 M.P**	**Class 2 M.P.**
Chl a	0.80	0.39
Chl b	0.79	0.30
a/b	0.77	0.50
K	0.58	0.70
Ca	0.63	0.36
Mg	0.71	0.31
Cd	0.55	0.21
Ni	0.73	0.57
Cu	0.69	0.76
Zn	0.32	0.53

The modelling power results show that Mg has little influence on Class 1, and Cd in the epicormic growth appears to have little importance for Class 2. The MCL plot (Fig 3.3) indicates the presence of two class types that have some degree of overlap. It is quite clear that sites 2, 3, 5 and 14 (good growth sites as classified for the 1986 foliar data) are separate from sites 4, 6, 8, 10, 12, 13 and 15 (poor growth sites as classified for the 1986 foliar data). Thus, on the basis of measuring the chlorophyll and nutrient levels of the one year old epicormic and non-epicormic growth a classification is obtained that has some similarity to the 1986 foliar data.

The variables that are contributing to the two classes for both the epicormic and non-epicormic growth are, the chlorophylls, potassium, calcium, zinc, nickel and copper. Magnesium is contributing more so for the epicormic growth in Class 1 and for the non-epicormic growth in Class 2.

Fig. 3.3 Multiclass Plot For the 1987 Foliar Data Showing the Class Structure.



The result from the 1987 survey reveals that there are no statistically significant differences between the chlorophyll levels and the trace metal levels for the epicormic and non-epicormic growths. This was slightly surprising since visually the epicormic growth appears to be much greener on the trees compared to the non-epicormic growth. This appearance may be due to there being a greater abundance of the epicormic growth in severely stressed trees.

CHAPTER 4

4. SURVEY OF A PAIR OF TREES AT SITE 15 OF THE AFAN 1 FOREST.

Part of the problem at the Afan Forest appears to be that some of the trees seem to be more capable of surviving the conditions encountered at the sites than others. The initial survey (Chapter 2) showed marked differences in the growth of the trees at the various sites. This difference in growth may be attributable to increased stress at higher elevations [Treshow and Anderson (1989)], since the poor growth sites are to be found at higher elevations. In the review on forest decline by McLauglin (1985)], some workers have suggested that there are genetic differences between trees of the same species in the same forest where some are growing very strongly and others are showing the signs of decline. At many of the Afan 1 forest sites, both the good and poor growth, there are pairs of trees that exhibit marked differences in growth. These pairs are easily observed since one of the pair is displaying strong healthy growth and the other is stunted with poor foliage. Generally these pairs are of the same age and come from the same seed stock [Danby (Pers. Comm.) (1987)].

At site 15 there is one such pair of trees, each from the same seed stock and of the same age. One of the pair is healthy, exhibiting strong growth with good foliage and the other is stunted with poor foliage. Since the initial survey in Chapter 2 revealed significant differences in potassium and calcium levels for the good and poor growth sites, it was decided to monitor the levels of K, Mg, Ca, Cu, Ni, Zn and chlorophylls 'a' and 'b' over a number months spanning summer, autumn and winter.

4.1. Materials and Sampling Procedure.

Two cuttings were collected from each of the pair of trees at site 15 on the first Monday of each calendar month from June 1987 to January 1988. The foliar samples were removed from branches approximately six feet from the ground. The sampled trees were approximately 27 years old. Only the present (one year old) needles were analysed. The needles were carefully removed from the branches and were randomised by 'bagging and shaking' prior to analysis. Three samples were used for each month.

For the chlorophyll analysis, fresh needles were used and the chlorophyll levels determined using the SCOR-UNESCO equations (Chapter 2). Metal determinations were carried out as set out in Chapter 2 except that a Philips PU 9150 AAS was used. The results for the Good growth tree are given in Table 4.1 and the results for the Poor growth tree are given in Table 4.2.

Table 4.1. Chlorophyll (fresh weight) and Metal Levels (dry weight) for the Good Growth Tree (mg kg^{-1})

Month		Chl a	Chl b	K	Ca	Mg	Ni	Cu	Zn
Jun	Mean	337	98	6887	1943	1132	7.5	1.9	19.0
	sd	78.2	22.5	199.7	222.2	33.1	1.91	0.52	1.23
Jul	Mean	648	176	5240	1150	769	5.0	2.0	19.2
	sd	85.8	41.3	249.5	116.8	31.2	1.09	0.41	1.11
Aug	Mean	609	175	5176	2176	1333	6.7	5.2	83.0
	sd	55.0	12.1	239.5	158.4	65.6	1.42	0.83	1.21
Sep	Mean	802	262	3883	1262	777	3.9	2.0	38.8
	sd	136.7	44.4	339.2	176.6	13.2	0.72	1.01	3.23
Oct	Mean	1163	360	3975	1956	668	6.0	6.0	26.0
	Low	102.4	80.9	234.7	222.5	31.2	0.44	1.53	7.64
Nov	Mean	1338	427	4174	1560	1049	5.0	3.0	13.0
	Low	123.2	56.3	210.3	229.1	30.2	0.81	0.46	7.2
Dec	Mean	931	268	4174	1560	763	6.0	2.0	52.0
	Low	90.7	67.1	269.9	237.4	25.2	1.81	0.62	3.12
Jan	Mean	762	232	4149	1480	1178	4.0	1.5	30.0
	Low	145.5	37.2	224.4	131.3	50.7	0.72	0.52	1.77

0.0 = below detectable limit

Table 4.2. Chlorophyll (fresh weight) and Metal Levels (dry weight) for the Poor Growth Tree (mg kg^{-1})

Month		Chl a	Chl b	K	Ca	Mg	Ni	Cu	Zn
Jun	Mean	292	92	6621	1216	707	8.1	16.2	64.9
	sd	146.0	49.8	132.5	224.6	63.2	0.96	0.78	1.66
Jul	Mean	476	141	4455	1139	981	5.0	3.8	51.4
	sd	130.8	49.8	132.5	224.6	63.2	0.96	0.78	1.66
Aug	Mean	771	214	1653	916	519	2.6	3.3	113.0
	sd	106.2	81.0	182.4	171.1	50.2	0.82	0.56	9.01
Sep	Mean	885	276	2100	700	800	6.0	2.0	60.0
	sd	17.8	101.3	106.7	211.8	17.8	0.78	0.35	2.55
Oct	Mean	859	283	1949	1560	873	7.8	4.0	20.0
	sd	139.1	122.7	179.8	177.8	42.7	1.42	0.78	1.22
Nov	Mean	983	303	1349	1128	493	6.7	10.0	51.0
	sd	104.7	144.0	147.5	149.8	65.8	0.26	0.96	2.14
Dec	Mean	579	147	3088	742	757	3.0	1.9	77.0
	sd	105.9	51.7	186.5	209.6	54.5	0.14	0.70	1.71
Jan	Mean	595	207	3017	714	617	5.0	3.0	12.5
	sd	76.6	72.0	197.74	117.04	18.2	0.79	0.96	0.78

0.0 = below detectable limit

The values for each measurement were plotted against each month to give a graphical representation of the data to illustrate any trends over eight months that may be present for the pair of trees (Figs 4.1 to 4.8). Table 4.3 shows the t-tests between the variables for the good and poor growth trees over the eight month period

Table 4.3. Paired t-tests for the Good and Poor Growth Tree Pair at Site 15.

Var.	Good Growth Mean	Poor Growth Mean	Good Growth sd	Poor Growth sd	Corr.	Pooled Variance	d.f.	t-value	2-tail Prob.	Sig.
Chl a	823.75	680.00	318.31	234.17	0.7891	58810	7	2.071	0.077	*
Chl b	249.75	207.88	105.85	76.51	0.8280	6706	7	1.962	0.091	*
K	4707.00	3029.00	1024.75	1760.06	0.8220	1481717	7	4.361	0.003	***
Ca	1635.88	1014.38	358.61	302.31	0.3944	42758	7	4.794	0.002	***
Mg	958.63	718.38	244.42	169.87	-0.8132	-33763	7	1.720	0.129	n.s.
Cu	2.95	5.53	1.70	5.02	-0.0143	-1.221	7	-1.319	0.229	n.s.
Ni	5.51	5.53	1.27	2.03	0.0675	0.174	7	-0.015	0.988	n.s.
Zn	35.13	56.23	22.99	31.67	0.7237	527.1	7	-2.731	0.029	**

90% *
95% **
99% ***
n.s. not significant

Chlorophylls 'a' and 'b' were found to be significantly higher in the good growth tree (90%) for the eight month period, while potassium and calcium were found to be significantly higher (99%) in the good growth tree over the same period. Zinc was found to be significantly higher in the poor growth tree over the eight months (95%). There were no significant differences encountered for the other metals measured in both trees. The higher levels of potassium and calcium are consistent with the findings of chapter 2, i.e. the good growth sites had significantly higher levels of potassium and calcium in the year 1 needles. The significantly lower (90%) chlorophyll levels of the poor growth tree may be a result of it performing less well in its environment compared to its' healthier partner.

There is a clear pattern in the monthly variation of both the chlorophyll 'a' (Fig. 4.1) and the chlorophyll 'b' (Fig. 4.2). For both trees there is a clear maximum in November for chlorophyll 'a' and chlorophyll 'b'. Some workers examining the pigment levels in Norway spruce have found that the chlorophyll levels peak in spring and winter [Staglfelt (1927), Zacharowa (1929). Atanasiu (1968) followed the trends of chlorophyll concentrations in various conifers from October to April and found that the highest concentrations were

obtained at the end of November. This agrees with the trends found for both trees. Figures 4.1 and 4.2 show that generally the good growth tree has the higher levels of chlorophyll 'a' and 'b'.

Figure 4.3 shows the variation in the potassium levels for the pair of trees over the eight month period. From June to January there is a marked decrease in the potassium levels for both trees. The decrease in the potassium levels throughout the period corresponds quite well with the results of Wyttenbach and Tobler (1987) who found the potassium levels in the younger needles of Norway spruce, decreased from June to August. By calculating the percentage changes in the potassium levels from June to January it is found (using the July figure as 100%) that the good growth tree has lost 37.8% of its potassium while the poor growth tree has lost 54.4% of its potassium.

The copper levels are shown in Fig. 4.4, the poor growth tree showing a marked decrease from June to January; a loss of 81.5% compared to its July figure. The good growth tree shows a much smaller decrease in its level of Cu, a loss of 21.1%. The variation in the calcium levels for the two trees is shown in Fig. 4.5. Here there is a general decrease in the calcium levels over the eight months. The good growth tree has lost 23.8% of its calcium (compared to its June figure) while the poor growth tree has lost 41.3% of its calcium.

The trend in the magnesium levels for the pair of trees is shown in Fig. 4.6. The poor growth tree has lost 12.7% of its magnesium while the good growth tree appears not to have lost any magnesium, there is in fact a slight gain (4.1%). With the nickel levels (Fig. 4.7), there is a general decrease in the level of the metal over the eight months for both trees. The good growth tree has lost 46.8% compared to its June amount and the poor tree has lost 38.3%. The good growth tree appears to have gained 57.9 % zinc

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Fig. 4.1 Chlorophyll 'a' Levels (mg kg^{-1} fresh weight) for the Pair of Trees at Site 15.

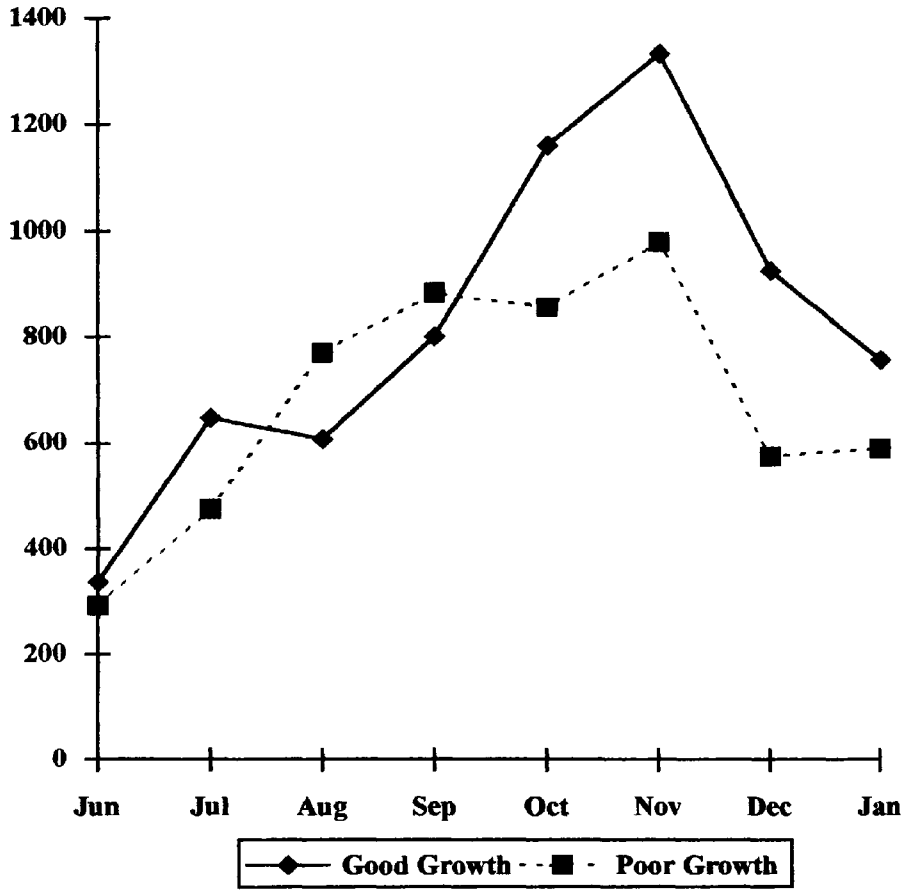


Fig 4.2 Chlorophyll 'b' Levels (mg kg^{-1} fresh weight) for the Pair of Trees at Site 15.

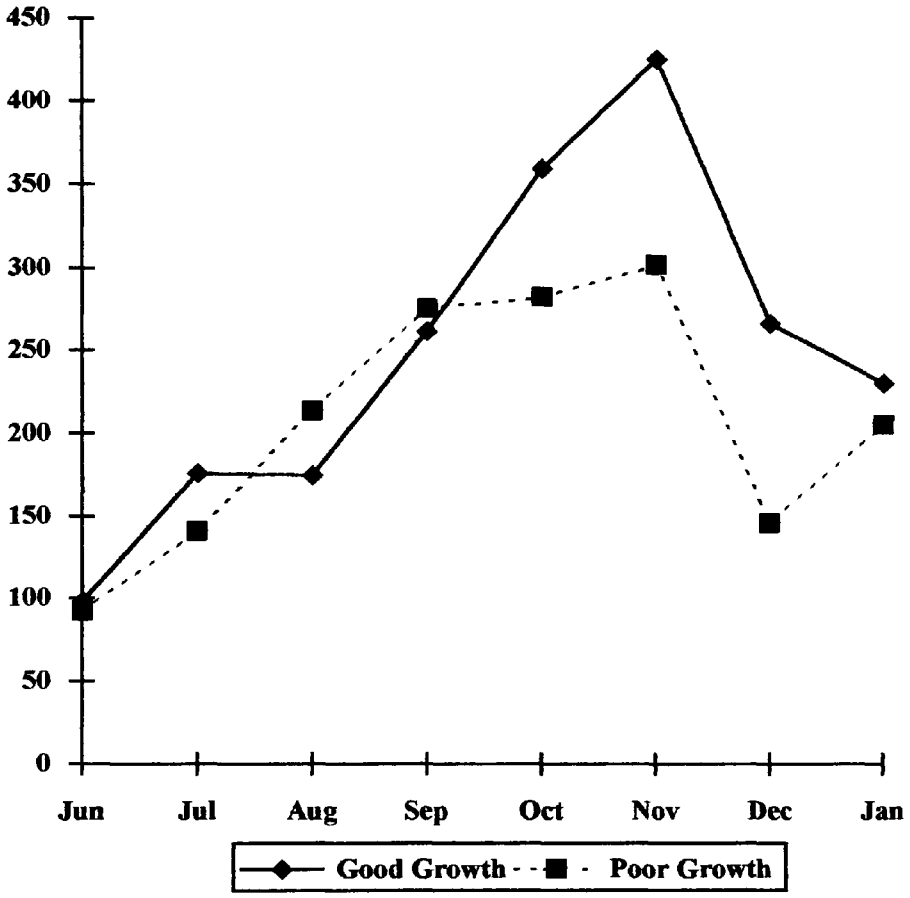


Fig 4.3 Potassium Levels (mg kg^{-1} dry weight)for the Pair of Trees at Site 15.

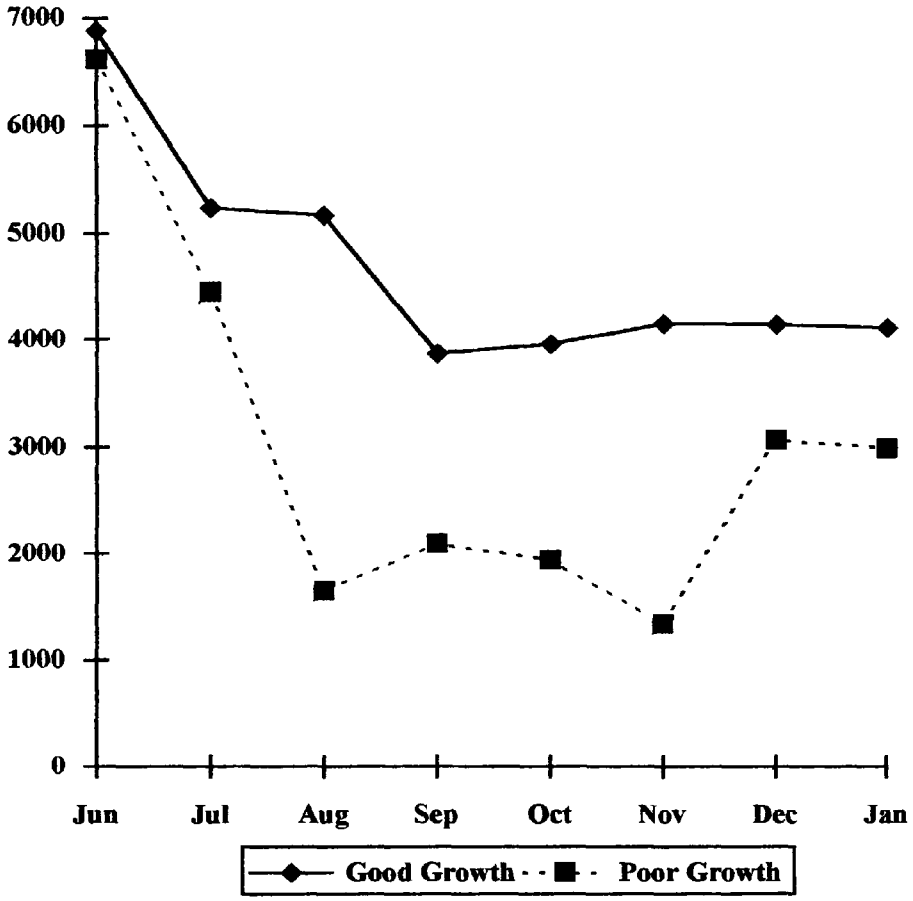


Fig 4.4 Copper Levels (mg kg^{-1} dry weight) for the Pair of Trees at Site 15.

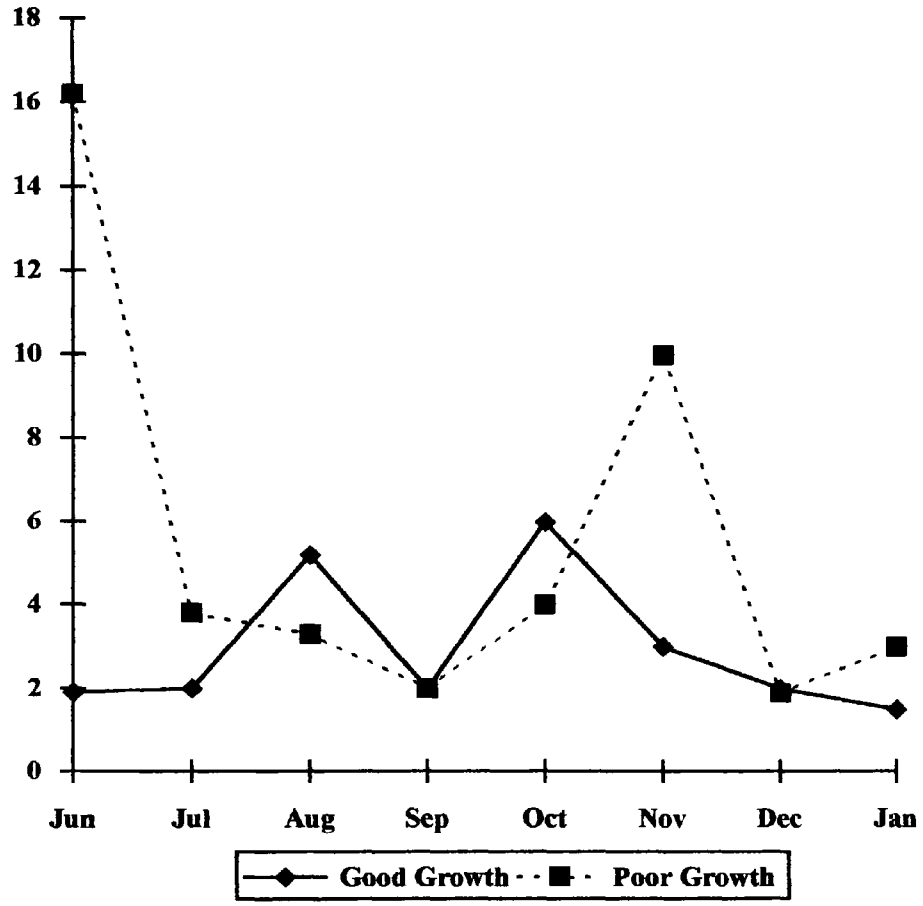


Fig 4.5. Calcium Levels (mg kg^{-1} dry weight) for the Pair of Trees at Site 15.

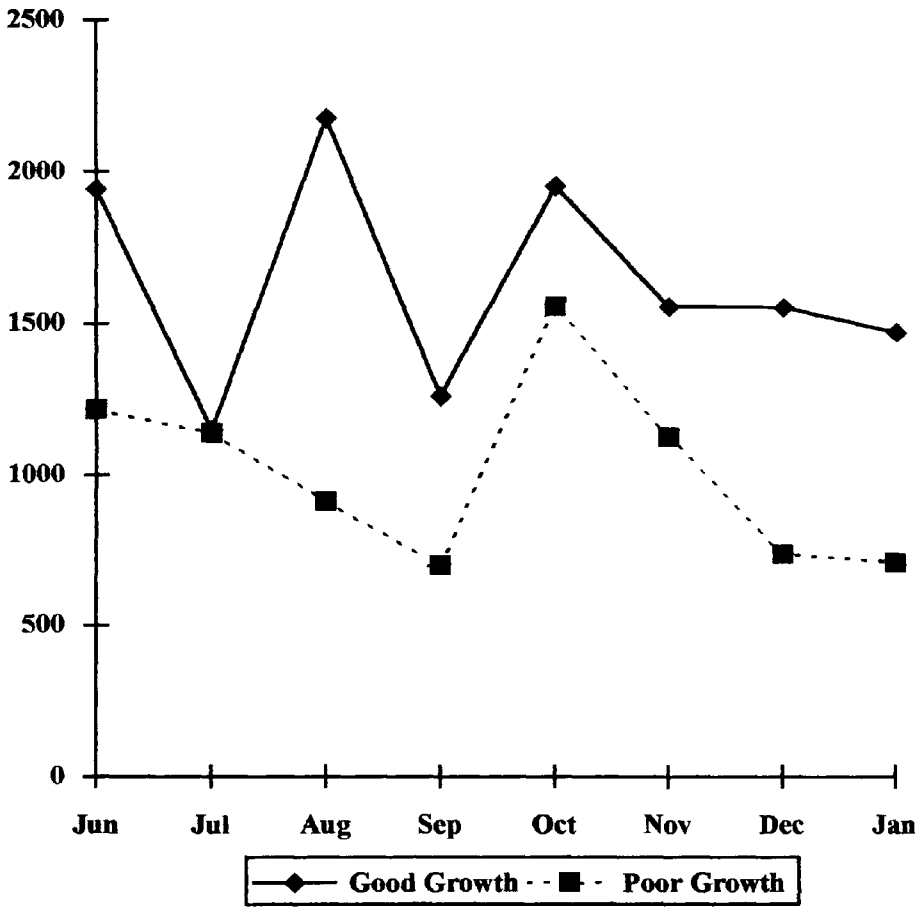


Fig. 4.6. Magnesium Levels (mg kg^{-1} dry weight) for the Pair of Trees at Site 15.

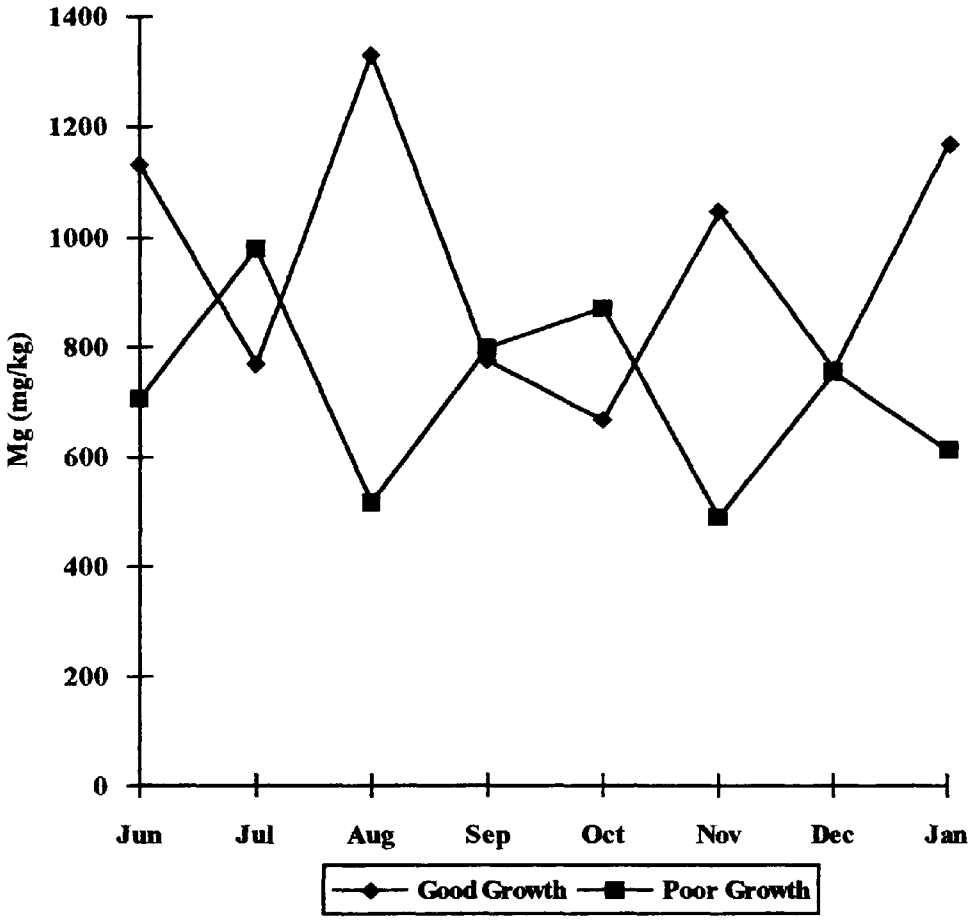


Fig. 4.7. Nickel Levels (mg kg^{-1} dry weight) for the Pair of Trees at Site 15.

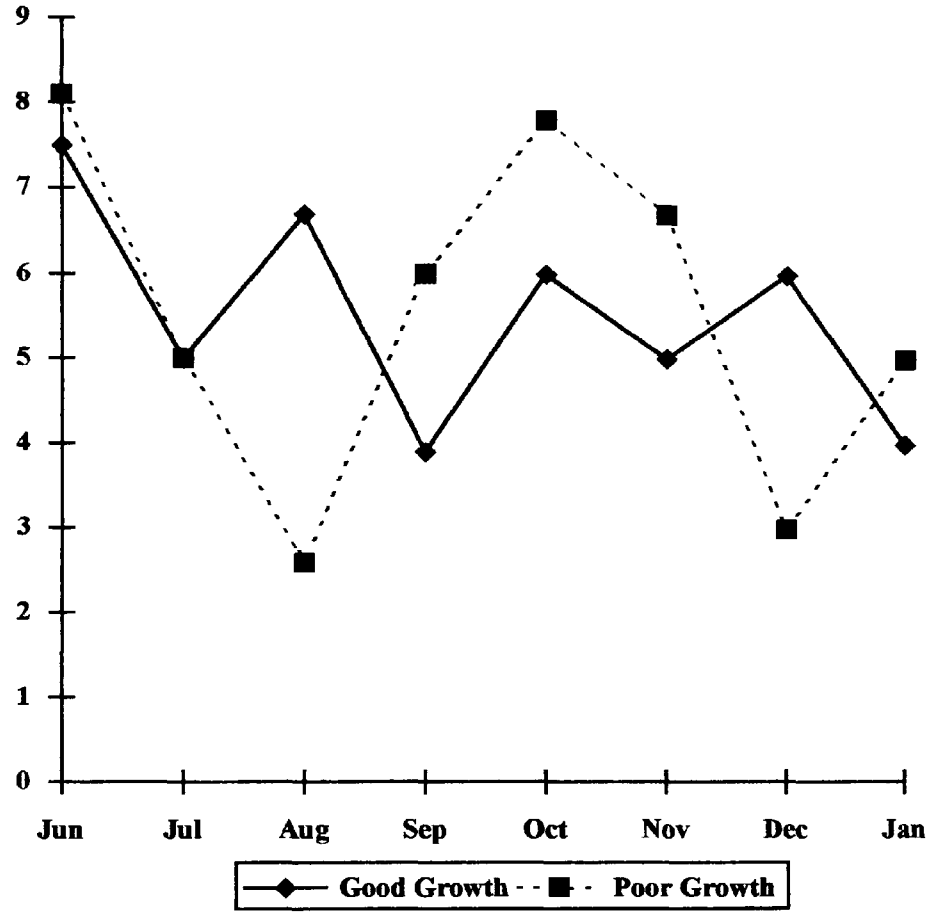
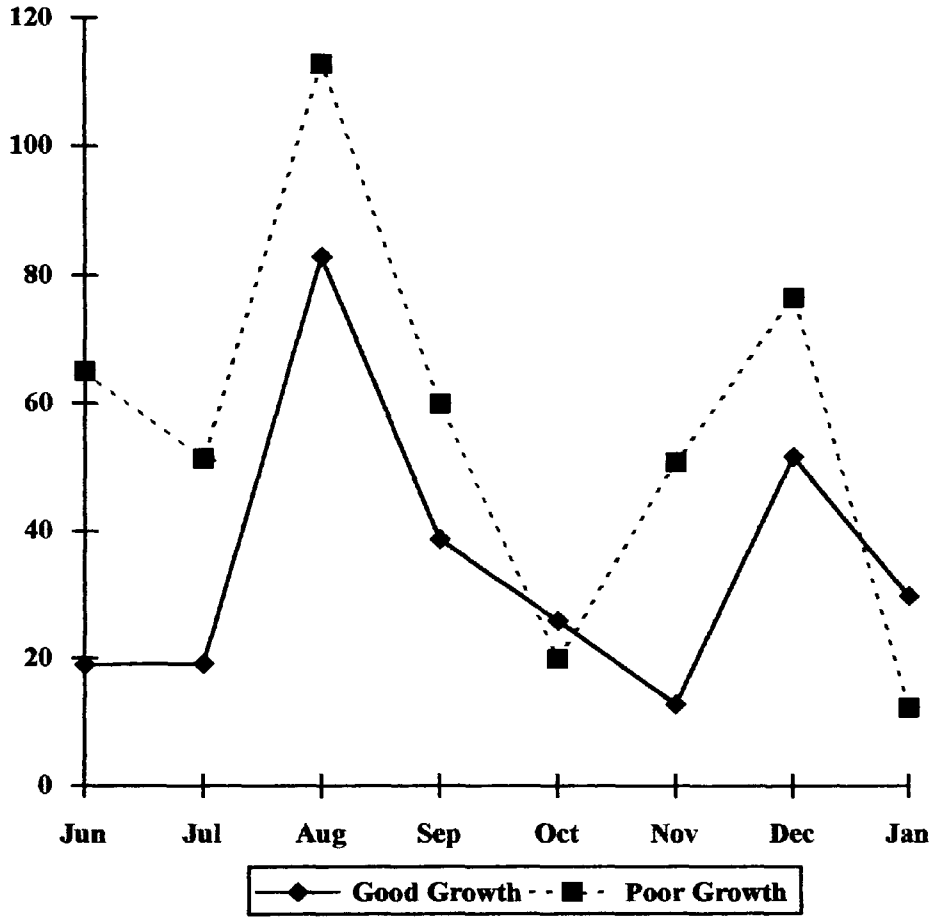


Fig 4.8. Zinc Levels (mg kg^{-1} dry weight) for the Pair of Trees at Site 15.



4.2 Peroxidase Assay.

McLaughlin (1985) suggests that there may be genetic differences between trees that are growing under the same conditions, but some are surviving quite well, whilst others are exhibiting the signs of forest die-back. Some enzyme systems have been suggested to be an indication of genetic differences within the same species. One such enzyme is peroxidase. Peroxidase occurs as isoenzymes and can be used for the identification of individual plants [Baaziz and Saaidi (1988)] and as a means for determining the genetic variability of populations of plants [Proctor et al (1989)].

Peroxidase enzymes are widespread throughout the plant kingdom. Their major function is the oxidation of molecules at the expense of hydrogen peroxide. They play a role in four physiological processes; auxin catabolism, lignin formation, some respiratory processes and defence mechanisms [Baaziz (1989)]. Peroxidase has been shown to stiffen the cell wall by catalysis of the oxidation of the cell wall phenolic compounds to form the more hydrophobic biphenyls, polymers or quinones, any of which could possibly protect the cell wall polysaccharides against attack by enzymes or water [Spanu and Bonfante-Fasolo (1988)].

Peroxidase has been used as a marker of stress situations in plants. Such situations can occur in many plant-pathogen interactions where peroxidase-requiring compounds such as phenols, lignin and suberin are laid down in the cell wall and act as a physical barrier against microbial attack [Spanu and Bonfante-Fasolo (1988), Baaziz and Saaidi (1988), Arora and Bajaj (1985)] and infections [Flott et al (1989)].

Thus, increased peroxidase activity has often been observed following a plant-pathogen interaction [Flott et al (1989)]. It has been shown that plants inoculated with selected

viruses or fungi exhibit increases in peroxidase activity and there is often associated with this the appearance of new isozymes [Yamamoto et al (19778].

Changes in isoperoxidase patterns after inoculation have often been reported. This may be due to only one kind of peroxidase being affected. Some work seems to indicate that there is no change in the isoperoxidase patterns [Gaspar et al (1982)]. It may be possible that airborne pollution can cause similar effects to those reported by workers involved with plant pathogens.

Peroxidase also occurs as isoenzymes. The isoenzymes of peroxidase have been used for the identification of individual species and as a means of determining the genetic variability of populations [Proctor et al (1989)]. The presence of some of the isoenzymes or their lack may indicate some effect due to pollution. Peroxidase activity has increased in plants that have been exposed to ozone [Curtis and Howell (1971), Declaire et al (1984)]. Ozone is a very reactive component of photochemical air pollutants causing foliar injury in many plants.

4.3 Materials and Methods

Three sites (1, 5 and 6) were examined at the Afan forest, in June 1990. At these sites there are pairs of Sitka spruce, which though they are of the same age exhibit marked differences in their growth.

The trees at these sites were planted in 1968; the soil types at sites 1 and 5 being both peaty gley whilst site 6 is a deep peat [Coutts (Pers. Comm) (1990)]. Each of these trees has had grafts taken from them by the Forestry Commission. Since the peroxidase enzyme system may play some role in a plant's defence mechanism, peroxidase activity and isoenzyme pattern in these pairs of trees and their grafts were examined to see if

there are any difference between them. It was also decided to see if the peroxidase activity and isoenzyme pattern could be used as a possible marker to determine how well a tree is coping with its growing environment.

4.3.1 Leaf Extract Preparation.

Fresh needles (1.5 g) were excised from the branches (one year old) and ground in a chilled mortar and homogenised in 5 cm^3 of 0.1M pH 7.5 Tris-HCl buffer, containing 10% glycerol (v/v) and 10% (w/v) insoluble Polyvinyl pyrrolidone (PVP). The homogenate was filtered and centrifuged at 20,000 x g for 20 minutes at 4°C. The clear supernatant (Tris buffer extract) was used to determine the protein content and the peroxidase activity. The foliar material was sampled and analysed in the same day to avoid denaturation of the enzyme.

4.3.2 Electrophoresis.

Electrophoresis was performed on the enzyme extract according to Baaziz (1989). The gels were 2-16% gradient polyacrylamide. The tank buffer was 0.05M, pH 8.3 Tris-glycine buffer, diluted 1:2 just prior to use. The voltage was fixed at 170 V. The staining mixture was composed of 80 cm^3 of 0.1 M pH 5.0 acetate buffer, 20 cm^3 0.12M guaiacol (fresh) and 0.1 cm^3 30% hydrogen peroxide. The gels were incubated with the substrate solution for 20 minutes, washed and fixed in methanol-water-acetic acid 5:5:1, by volume.

4.3.3 Peroxidase Activity Determination.

The assay mixture for the determination of the peroxidase activity consisted of 1.0 cm^3 0.1M, pH 5.4 acetate buffer, 2 cm^3 30 mM guaiacol (fresh) and 0.1 ml of the Tris buffer extract diluted 1:10 with acetate buffer. The reaction was initiated by adding 0.05 cm^3 of 30% hydrogen peroxide. The increase in absorbance was monitored at 470 ηm for 6

minutes using a Cecil 5000 double beam spectrophotometer. Activity was expressed on a fresh weight basis (units of enzyme per gram of fresh weight) or as specific activity (units of enzyme per milligram of protein). One unit of enzyme was defined as the amount of enzyme that gave an absorbance change of 0.1 absorbance units in 1 minute. Protein determination was carried out according to the method developed by Lowry (1951) using bovine serum albumen as standard. The results of the protein determination and the enzyme assay are given in Table 4.3

Table 4.4. Protein determination and Peroxidase Assay Results for the Good Growth and Poor Growth Trees at Sites 1, 5 and 6.

	Protein Concentration				Peroxidase Activity			
Tree	**mg cm^{-3}**	**sd**	**mg g^{-1}**	**sd**	**SA***	**sd**	**Units g^{-1}**	**sd**
Good 1	7.93	0.33	26.44	1.09	41.03	0.83	1084.94	49.49
Good 5	5.83	0.31	19.45	1.02	3.72	0.84	71.70	14.50
Good 6	4.40	0.73	14.67	2.45	32.81	5.66	472.65	78.61
Poor 1	6.63	1.08	22.11	3.61	4.14	1.41	86.48	15.84
Poor 5	5.32	0.34	17.72	1.13	35.68	7.12	633.53	139.72
Poor 6	6.58	0.90	21.91	3.00	58.17	8.50	1252.94	86.62
GRAFTS								
Good 1	6.95	0.65	23.17	2.16	67.77	9.31	1540.03	215.06
Good 5	6.10	1.06	20.33	3.54	29.75	0.97	605.52	113.26
Good 6	3.68	0.59	12.28	1.98	18.09	4.33	213.49	17.78
Poor 1	5.33	0.59	17.78	1.98	17.71	3.44	310.12	49.26
Poor 5	9.67	1.27	32.22	4.22	44.49	18.24	1361.45	374.93
Poor 6	4.42	0.12	14.72	0.42	121.30	20.80	1785.19	309.45

* SA - specific activity: units per milligram of protein. All the results are the mean of three experiments.

The protein concentration obtained via the Lowry method was used to obtain the total protein concentration (mg per gram of fresh needles). The specific activity of the enzyme was determined by working out the number of enzyme units that were present in 1 cm^3 of the plant extract. This was determined by first calculating the number of units from the change in absorbance per minute, since a change of 0.1 absorbance units in one minute would equal one unit of enzyme. This value would then have to be multiplied by a

dilution factor of 315. 0.1 cm^3 of enzyme extract was diluted to 1 cm^3 with acetate buffer. 0.1 cm^3 of this was then used for the assay and to it was added 2 cm^3 of guaiacol, 1 cm^3 of acetate buffer and 0.05 cm^3 of 30% hydrogen peroxide solution. This gave a total volume in the cuvette of 3.15 cm^3 that equates to a 31.5 times dilution of the extract that was then diluted ten times so the total dilution factor is 315 times. The units of enzyme per mg of protein is simply calculated by multiplying the amount of protein per gram of fresh needles by the specific activity of the enzyme.

The enzyme assay result reveals considerable variation in the specific activity of the peroxidase. Of the trees that are exhibiting strong growth the specific activity of the peroxidase is about ten times lower for site 5 compared to sites 1 and 6 (4 and 40 respectively). For the trees exhibiting poor growth, site 1 has a specific activity that is about nine times lower than for site 5 and about fourteen times lower for site 6. The grafts all exhibited activity that was generally higher than the trees from which they were removed. This change in peroxidase activity may be a reflection of improved growing conditions after grafting.

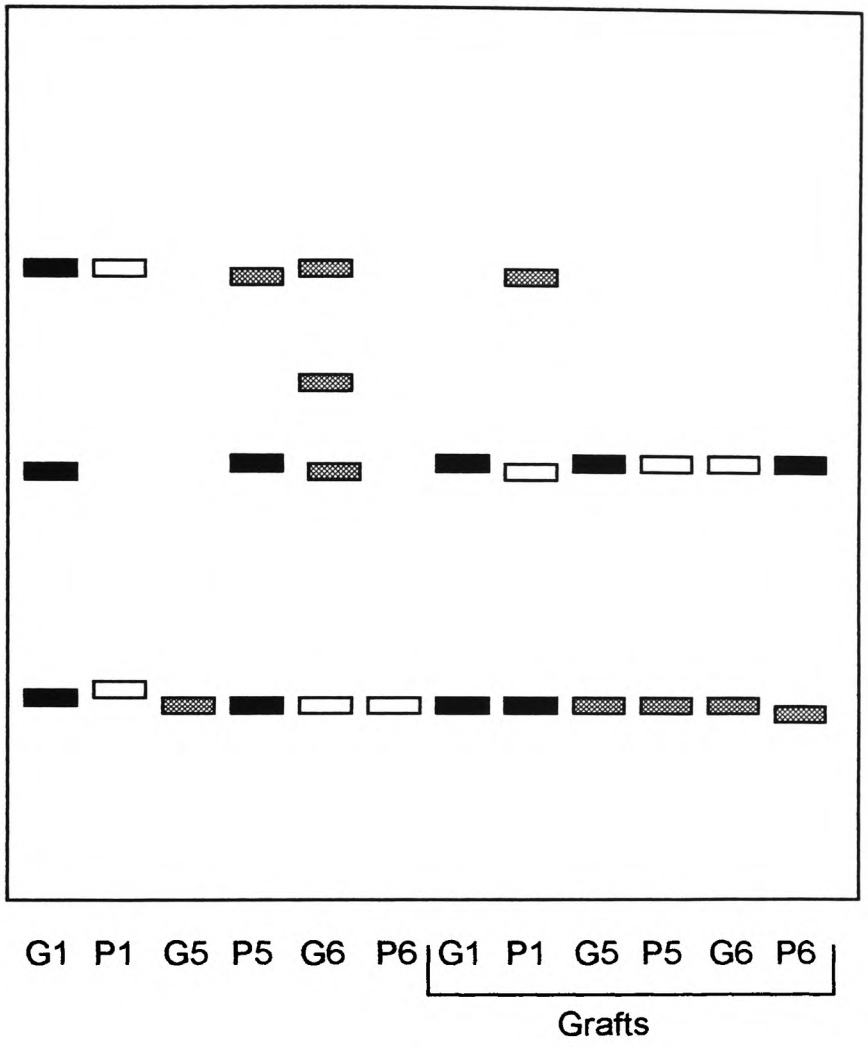
Declaire et al (1984), showed that peroxidase activity increased as a function of the concentration of ozone. Ozone has been shown to affect the permeability of plant cells. Other air pollution such as sulphur dioxide has also been implicated as an airborne pollutant [Pierre and Queiroz (1981) Ormrod (1984)]. Similarly, the oxides of nitrogen are also known to cause a decreased growth rate in trees [Wellburn (1989)]. Therefore it is quite conceivable that the various forms of airborne pollution may damage plant cells and in turn cause increase in the activities of the peroxidase enzymes.

Gel electrophoretic separation of the crude enzyme extracts revealed a pattern of the isoperoxidases with all the trees looked at (Fig. 4.12). All the trees had band 1 and most

band 2. Band 3 was only observed in the following trees: Good Growth at sites 1 and 5 and Poor Growth site 5 and Poor Graft from site 1.

Peroxidases have been used as an aid in the determination of different plant cultivars and as a means of assessing the genetic variability of populations [Proctor et al (1989), Baaziz (1989)]. The banding in Fig. 4.12 shows that there are differences between the trees at the three sites and their grafts. However is not possible with these results to conclude that different bands are due to any genetic differences. It would be expected that the grafts should have similar banding patterns to the trees they were removed from (since they are genetically the same) but the results show otherwise. This difference in the banding patterns may be due to the trees coping with any environmental stresses in different ways.

Fig. 4.9. Anodic Separation of Peroxidase Isoenzymes Via PAGE.



CHAPTER 5

5. Chromatography of Plant Pigments.

Plant pigments have been routinely analysed using high performance liquid chromatography (HPLC). HPLC lends itself to this since it allows the separation of the individual pigments and thus allows them to be evaluated individually. To date the only plant pigments that have been analysed using Supercritical Fluid Chromatography (SFC) are the orange\yellow carotenoids [Chapman (1988), Frew et al (1988), Shepherd (1989)].

5.1 Determination of the Accuracy of the SCOR-UNESCO Equations Using A Multivariate Calibration Method.

In the earlier part of this work, the SCOR-UNESCO equations were used to determine the chlorophyll levels. These simultaneous equations can be determined and provide values for the chlorophyll 'a' and 'b' concentrations in a sample of pigment extract from a plant. If other observed pigments are present they may absorb at one or more of the wavelengths used and interfere with the results determined. Chromatographic techniques are superior since they facilitate the assessment of the individual pigment concentrations by separation and analysis. HPLC is the technique often applied to porphyrins such as the chlorophylls because it can separate the compounds and detect them in the visible part of the spectrum. However, HPLC is time consuming, both in preparation of individual standards and actual analysis times. The SCOR-UNESCO equations on the other hand, are rapid in both sample preparation and analysis, and this method can lend itself to field work with portable spectrophotometers. Multivariate statistical methods may provide a calibration technique to relate the levels of chlorophylls 'a' and 'b' determined via the

SCOR-UNESCO equations and the HPLC method.

In using HPLC to determine the chlorophyll levels a suitable method must be found or developed. There have been many publications covering the HPLC of chlorophylls [Eskins et al (1977), Schoch et al (1978), Braumann and Horst-Grimme (1981), Burke and Arnoff (1981) and Shioi et al (1983)]. Many of these methods use methanol as one of the eluting solvents. Some researchers have suggested that methanol can cause allomerization of chlorophyll 'a' and 'b' [Strain and Svec (1966), Hynninen (1979a and 1979b)]. Owing to their exceptional lability, the chlorophylls are susceptible to a number of chemical transformations that yield various products and isomers. This lability stems from the high reactivity of the moderately strained isocyclic ring in the chlorophyll molecules.The allomerised chlorophylls possess different retention times to the 'normal' chlorophyll 'a' and 'b' and therefore care must be taken to avoid this problem.

Another aspect to this problem is the provision of quality chlorophyll standards. Iriyama et al (1974) successfully precipitated a mixture of chlorophylls 'a' and 'b' from a dioxane complex. The chlorophylls were then separated by standard chromatographic techniques. The dioxane precipitation was also carried out by Scholz and Ballschmiter (1981). They used a mixture of acetonitrile and water as eluent for separating a mixture of chlorophyll 'a' and 'b' via a preparative liquid chromatography procedure.

In this calibration problem there are identifiable dependent variables. These are chlorophylls 'a' and "b' concentrations. The independent variables are the absorbance values. The calibration is formed by using the chlorophyll concentrations obtained by the HPLC method, in conjunction with a series of absorbance readings (which include the values necessary for the SCOR-UNESCO equations).

The classical method of mulitvariate analysis, that is Multivariate Regression (MR) requires more objects (experimental runs) than variables (the measurements for these runs) [Geladi (1988)]. If a situation arises where there are more variables than objects a step-wise regression approach must be adopted. This has the disadvantage that variables containing valuable information may be eliminated. However, the use of Partial Least Squares (PLS) regression can overcome this, since all the information can be retained in the problem by extracting 'latent' variables that are linear combinations of the original variables. These 'latent' variables describe the maximum predictive variance of the data and provide the 'best-fit' to the model. In a PLS problem, the data can be divided into two blocks, namely X and Y.

Partial Least Squares has been used many times in calibration problems [Smilde and Doornbos (1992), Seasholtz and Kowalski (1992)]. The technique was developed by Herman Wold in 1966. The X block contains the absorbances observed on the samples. These have known concentrations of chlorophylls 'a' and 'b' that form the Y block. It may be necessary to classify objects and to predict their values of 'dependent' variables Y. The data set can also be divided in to a training set and a test set (Fig 5.1).

Fig. 5.1. Representation of the Training Set and Test Set Data.

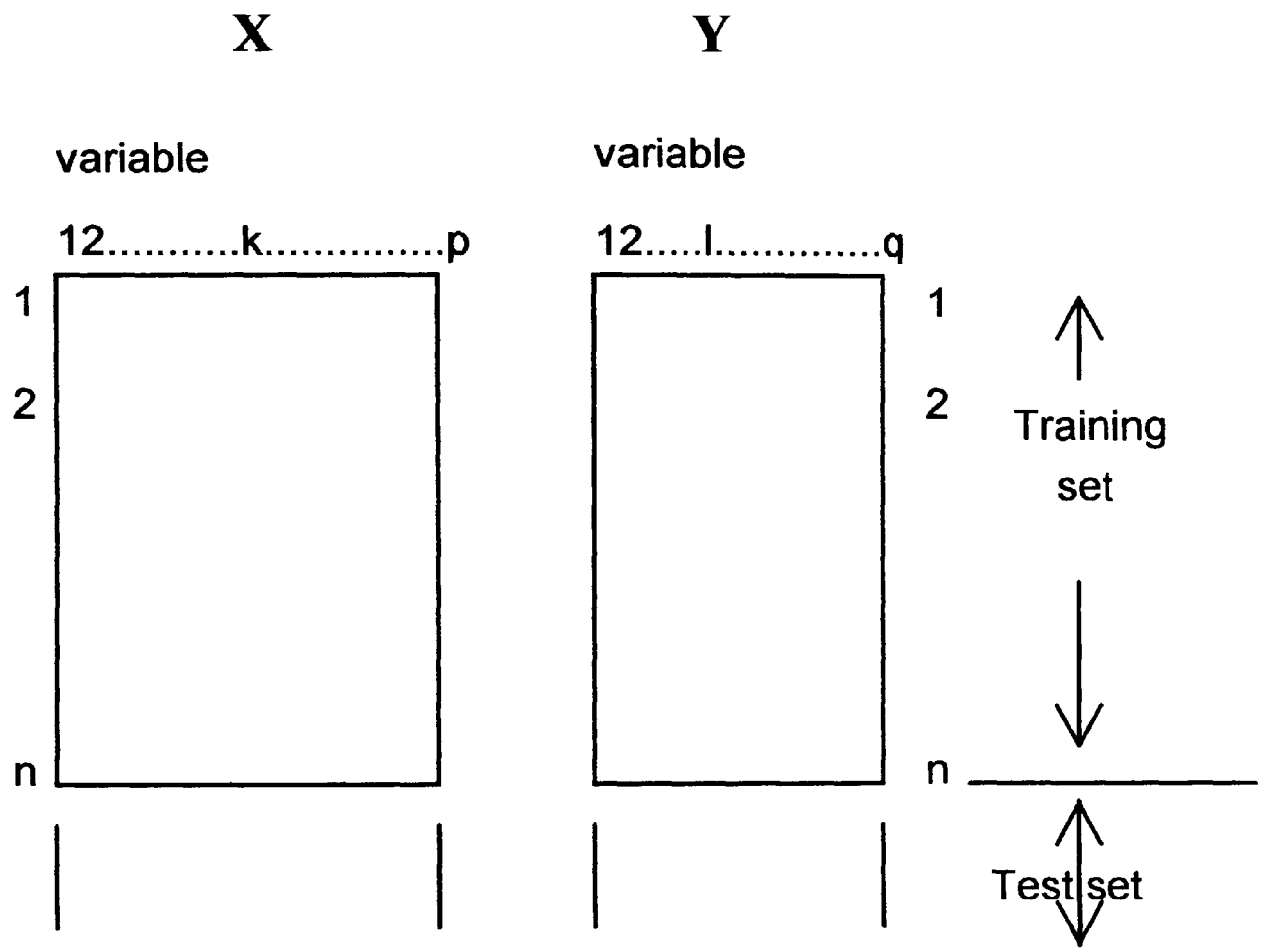
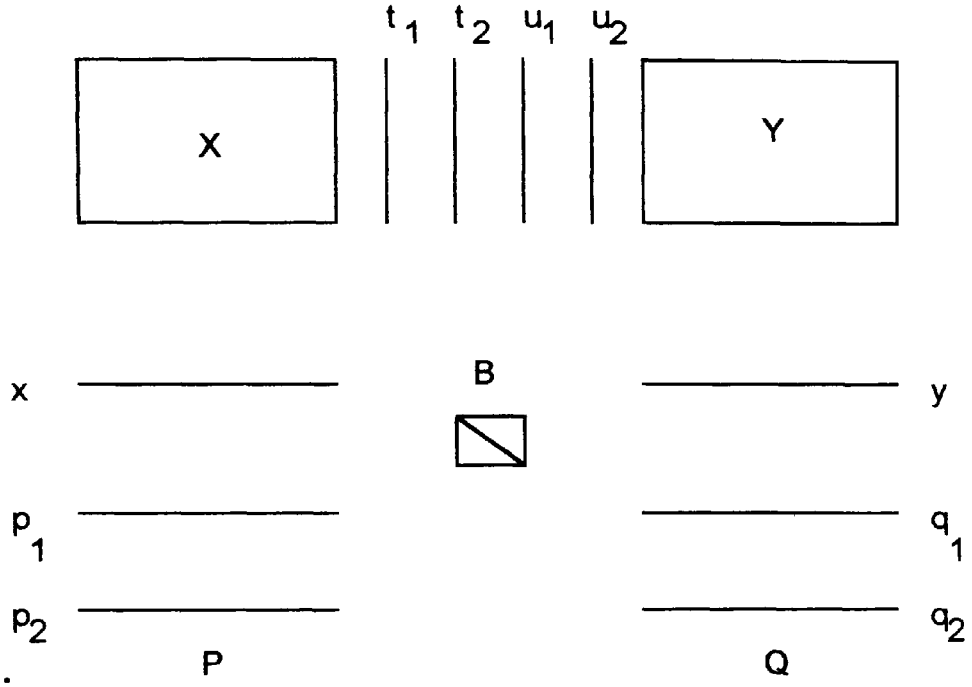


Fig. 5.2. PLS Model in Matrix Form



$$x = 1.\bar{x} + TP + E \qquad y = 1.\bar{y} + UQ + F$$

$$U = T.B + h$$

PREDICT

The PLS method produces a matrix T that is a projection of X and is calculated both to approximate X and to predict Y; the latter matrix is used to 'tilt' the PC plane to improve the relation between T and Y (Fig. 5.2) [Wold et al (1984)]. The PLS method has a further advantage in that it can handle several Y variables. This is achieved by having a separate space for the Y data and computing a projection U that is predicted by T at the same time it as approximates Y. Figure 5.2. Depict the PLS model in matrix form., the T and P matrices model the X block, while matrices U and Q model the Y block. The connection between the blocks is modelled as a relation between the U and the T matrices by means of a diagonal matrix B. The block residual matrices are E and F and the vector of the residuals for the inner relations is h [Wold et al (1984)].

5.2 Chromatographic Separation of Plant Pigments.

The dioxane precipitation used here was an adaptation of the method used by Iriyama et al (1974). The final separation of the chlorophyll 'a' and 'b" was achieved using cellulose preparative TLC. The concentrations of the chlorophyll 'a' and 'b' were determined using the molar absorptivity data of Vernon (1960).

Fresh privet leaves (50g) with the mid-rib were used for the extraction of the chlorophylls. The leaves were homogenised for 3 minutes with 250 cm^3 of acetone in a blender. The green juice was filtered through Whatman No. 1 to remove the debris. The dark green supernatent solution obtained was mixed with about 40 cm^3 of dioxane. Deionised water (approximately 60 cm^3) was added dropwise whilst stirring. The mixture was then left in a freezer for between 1 to 2 hours. After this time, a mixture of chlorophyll 'a' and 'b' precipitated out.

The chlorophylls were collected on sinters and washed with a little ice -cold petroleum ether (or hexane) to remove any associated carotenoids. The yellow/green solution that is left after the filtration gives a good indication that the chlorophylls have been removed. The chlorophylls were then washed off the sinters using the minimum amount of pure acetone. The separation of the chlorophyll 'a' and 'b' was achieved using preparative TLC on cellulose plates (Machery and Nagel MN 300 HR) using 1% iso-propyl alcohol (IPA) in petroleum ether (40-60).

Analysis of the mixture of chlorophyll 'an' and 'b' was achieved on a Varian Vista 5000 Liquid Chromatograph with a Varian Vista 402 data station and a Varian UV 50 detector set at 436 ηm using an isocratic system of 100% acetonitrile with a flow rate of 1 cm^3min^{-1}. Separation was achieved on a Chrompak, Chromspher C-18 reversed phase 3μm analytical column (100 x 3 mm). This method was then successfully employed in the separation of chlorophyll 'a' and 'b' in a Sitka spruce extract in acetone (Fig 5.3).

5.3. Absorbance and HPLC Results.

Twelve extracts of Sitka spruce were used in forming the model using the absorbances at 29 different wavelengths (Table 5.1 A and Table 5.1 B). Six of these samples were doped with chlorophyll 'a' and 'b' extract and are designated D1 to D6. The concentrations obtained using HPLC and the SCOR-UNESCO are given in (Table 5.2 A). Sample D6 and B6 were used as the test set.

Table 5.1 Absorbance Measurements for the Doped Extracts.

λ ηm	D1	D2	D3	D4	D5	D6
750	0.024	0.024	0.036	0.018	0.006	0.021
725	0.027	0.024	0.042	0.018	0.012	0.027
700	0.045	0.048	0.063	0.037	0.030	0.051
690	0.102	0.096	0.123	0.228	0.099	0.120
680	0.528	0.498	0.570	0.432	0.708	0.723
670	2.556	2.334	2.529	2.109	3.159	3.240
663	3.846	3.399	3.627	3.153	4.131	4.224
650	1.887	1.653	1.788	1.527	2.043	2.115
645	1.386	1.227	1.344	1.131	1.488	1.563
630	1.044	0.672	0.744	1.095	0.807	0.846
610	0.810	0.726	0.798	0.654	0.858	0.897
600	0.621	0.558	0.621	0.498	0.639	0.681
590	0.531	0.480	0.537	0.423	0.564	0.603
580	0.543	0.486	0.543	0.432	0.567	0.603
560	0.330	0.297	0.345	0.258	0.333	0.378
540	0.273	0.252	0.300	0.216	0.273	0.324
520	0.231	0.216	0.264	0.180	0.222	0.279
500	0.444	0.426	0.501	0.375	0.462	0.567
480	1.392	1.314	1.530	1.176	1.359	1.674
460	2.793	2.571	2.931	2.370	3.042	3.234
450	2.844	2.610	2.976	2.409	2.979	3.267
440	4.107	3.777	4.233	3.468	4.269	4.653
430	5.676	5.133	5.562	4.788	5.976	6.290
420	4.443	4.026	4.413	3.717	4.626	4.968
410	4.200	3.840	4.185	3.522	4.416	4.752
400	3.417	3.162	3.438	2.883	3.558	3.882
390	3.183	3.036	3.270	2.784	3.342	3.672
380	3.540	3.447	3.651	3.093	3.675	4.017
370	3.654	3.624	3.750	3.258	3.756	4.116

Table 5.2 Absorbance Measurements for the Non-Doped Extracts.

λ ηm	B1	B2	B3	B4	B5	B6
750	0.011	0.011	0.009	0.008	0.013	0.018
725	0.011	0.013	0.011	0.010	0.015	0.024
700	0.023	0.027	0.026	0.023	0.027	0.039
690	0.057	0.075	0.068	0.062	0.060	0.087
680	0.305	0.402	0.401	0.375	0.321	0.432
670	1.347	1.738	1.714	1.554	1.308	2.019
663	1.880	2.354	2.165	1.964	1.644	2.940
650	0.983	1.243	1.222	1.059	0.866	1.422
645	0.761	0.961	0.940	0.811	0.651	1.052
630	0.400	0.512	0.478	0.422	0.351	0.576
610	0.421	0.537	0.496	0.441	0.374	0.618
600	0.333	0.422	0.393	0.345	0.294	0.471
590	0.286	0.364	0.343	0.302	0.256	0.405
580	0.280	0.355	0.330	0.293	0.249	0.414
560	0.181	0.224	0.220	0.191	0.169	0.252
540	0.161	0.205	0.204	0.178	0.164	0.213
520	0.149	0.178	0.176	0.159	0.151	0.180
500	0.326	0.437	0.368	0.347	0.325	0.363
480	1.135	1.552	1.278	1.148	0.965	1.134
460	2.011	2.599	2.320	2.025	1.642	2.247
450	2.024	2.591	2.325	2.060	1.694	2.280
440	2.513	2.997	2.760	2.550	2.165	3.324
430	2.830	3.084	2.978	2.920	2.620	4.497
420	2.518	2.913	2.740	2.590	2.300	3.486
410	2.426	2.795	2.650	2.500	2.255	2.889
400	2.132	2.486	2.340	2.195	1.984	3.144
390	2.265	2.615	2.245	2.170	1.974	2.583
380	2.655	2.877	2.350	2.340	2.155	2.955
370	2.978	3.017	2.370	2.385	2.240	3.141

Table 5.3 Chlorophyll Levels (mg dm^{-3}) Determined via HPLC and SCOR- UNESCO.

	HPLC		SCOR-UNESCO	
	Chl a	**Chl b**	**Chl a**	**Chl b**
D1	45.90	10.70	41.60	9.80
D2	39.90	9.60	36.86	9.60
D3	44.20	11.40	39.00	10.70
D4	33.70	8.20	34.28	7.00
D5	49.70	11.60	44.89	11.89
D6	55.10	17.00	45.67	12.76
B1	21.30	8.50	20.17	6.94
B2	26.00	9.70	25.27	8.86
B3	21.20	7.20	23.13	9.31
B4	20.20	7.20	21.07	7.62
B5	17.70	6.20	17.87	5.60
B6	28.40	7.10	31.80	8.30

The calibration graphs for the HPLC of chlorophylls 'a' and 'b' are shown in Figures 5.4 and 5.5. The results of the calibration are given in Table 5.3.

Table 5.4. Calibration Data for Chlorophylls 'a' and 'b'.

Chl 'a' concentration (mg dm^{-3})				**Chl 'b' concentration (mg dm^{-3})**			
Conc.	**Av. Peak Area**	**sd**	**%sd**	**Conc.**	**Av. Peak Area**	**sd**	**%sd**
71.3	1562007	98728	0.63	23.6	333277	16974	5.1
49.9	1057856	18408	1.74	16.5	257556	7386	2.9
42.8	876878			9.3	138990	9800	7.1
21.4	439516	19391	4.40	7.1	77714	14257	18.3
14.3	337946	17635	5.30	4.7	43341	8967	20.7

Fig 5.3. HPLC Chromatogram of the Separation of Chlorophylls 'a' and 'b' From an Extract From Sitka Spruce Using a Chrompak, Chromspher C-18 reversed phase 3μm column (100mm x 3mm) using 100% Acetonitrile. λ436 ηm. 0 to 0.1 a.u.f.s

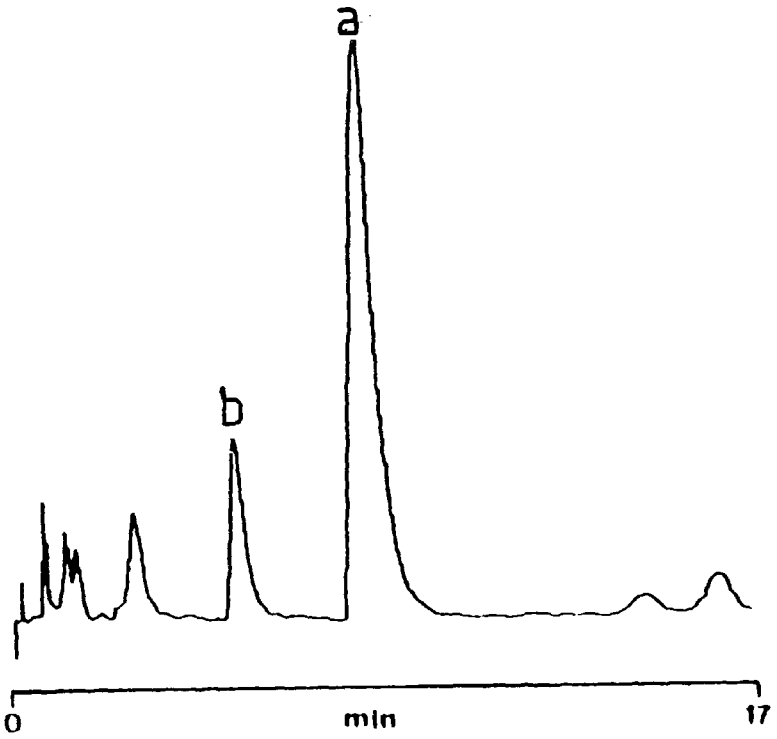
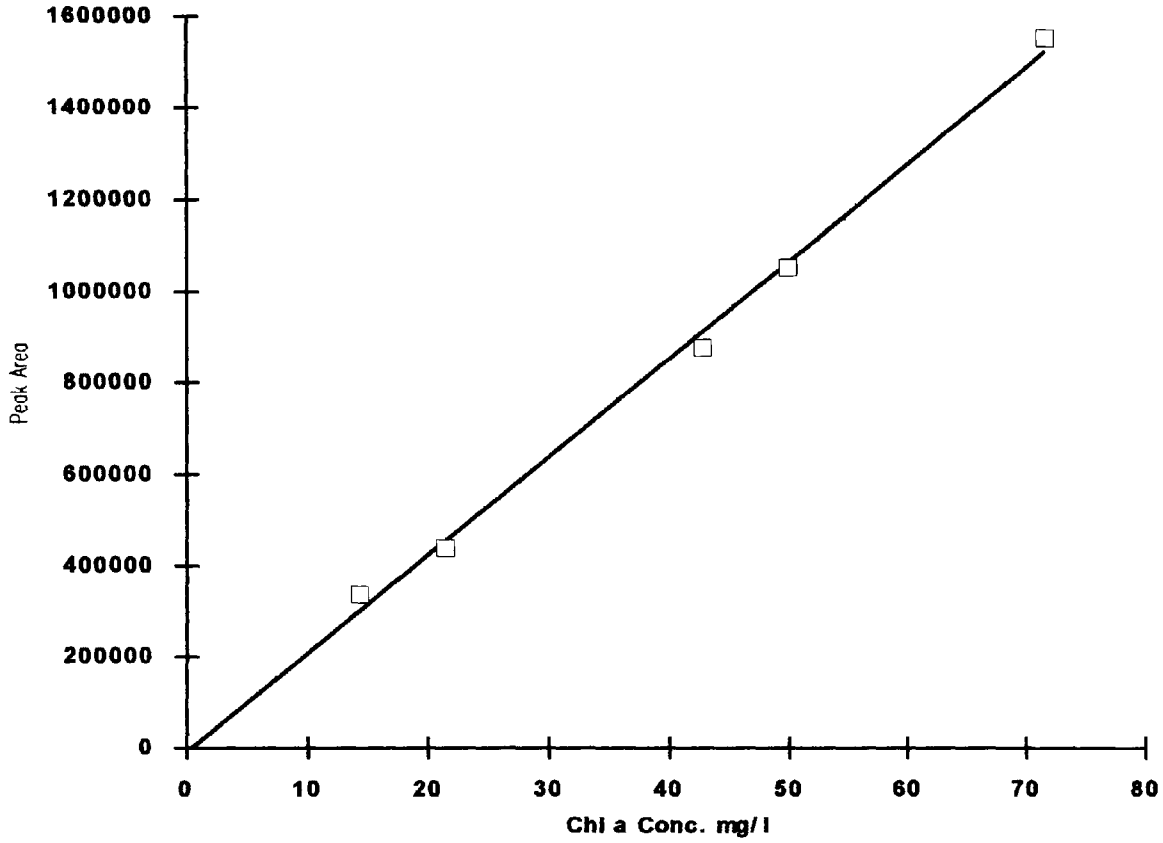
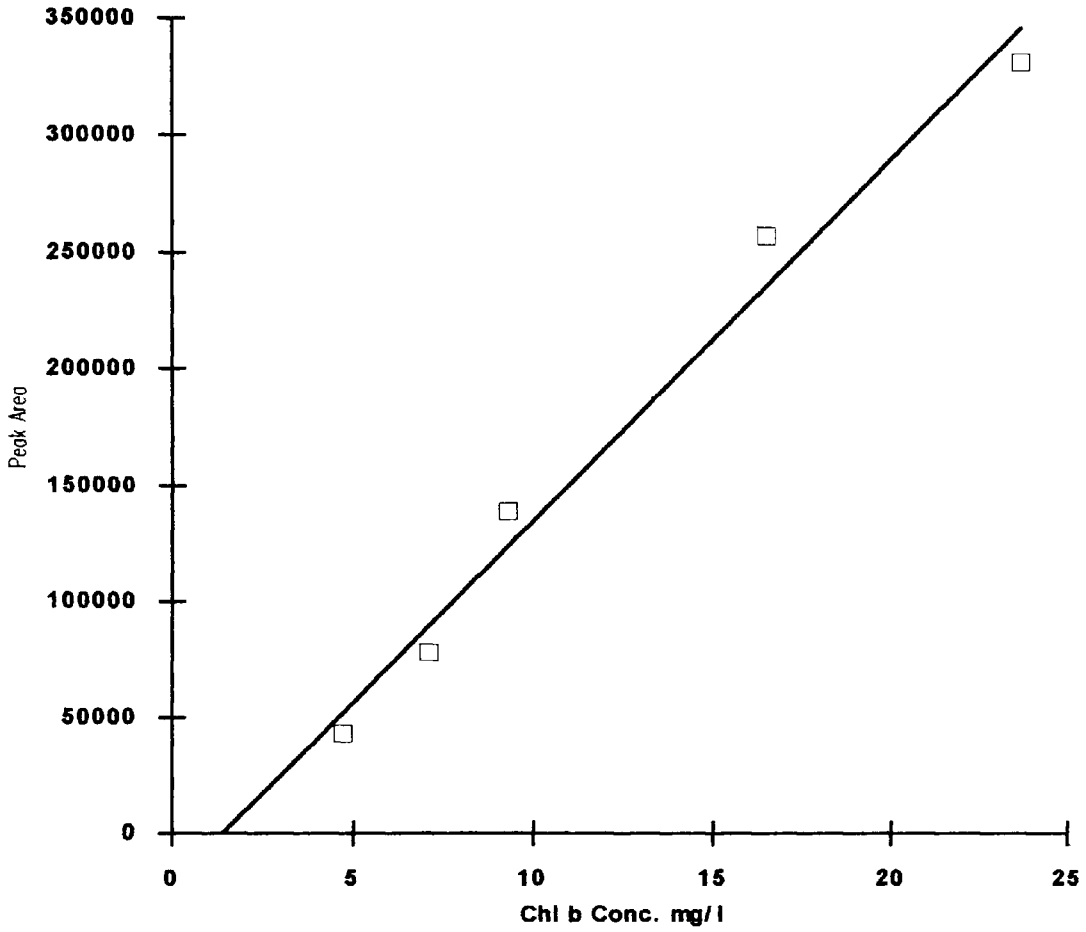


Fig. 5.4. Calibration Graph for Chlorophyll 'a' (Peak Area versus Chl 'a' Conc. mg dm^{-3})



	Chl a
Gradient	21613.6
Y Intercept	-8407
Correlation	0.998
Standard Error	37406.6

Fig. 5.5. Calibration Graph for Chlorophyll 'b' (Peak Area versus Chl 'b' conc. mg dm^{-3}



	Chl b
Gradient	15676.1
Y Intercept	-21699.9
Correlation	0.9909
Standard Error	19032.41

Detector response plots were obtained for chlorophyll 'a' and chlorophyll 'b'. These plots give an indication to the sensitivity of the detector and these are shown in figures' 5.5 and 5.6. The plot for chlorophyll 'b' shows the response to be very poor. The response of the detector for chlorophyll 'b' indicates non-linearity at low levels of chlorophyll 'b'. The detector response is determined by dividing the peak area by the concentration (for that particular peak area) which is then plotted against the peak area. An ideal response would be a horizontal straight line.

5.4. Results of the PLS Calibration.

Twenty-nine wavelengths were used to provide a model containing two Principal Components (these accounted for 91% of the variance in the data). The results of the training set together with the test set are given in Table 5.4.

Table 5.5 PLS Calibration Results for Chlorophyll 'a' and 'b' mg dm^{-3}

	Object	Chl a	Chl b
Training Set	D1	44.592	10.625
	D2	39.261	10.059
	D3	47.231	11.227
	D4	34.884	8.041
	D5	45.857	11.508
	B1	19.483	7.451
	B2	27.703	9.500
	B3	23.907	8.162
	B4	20.256	7.433
	B5	16.126	6.294
Test Data	D6	53.407	12.897
	B6	31.309	8.533

The predicted results of the PLS prediction for chlorophyll 'a' is quite close to the actual values given by the HPLC method. Figure 5.8 shows the correlation between the two sets of figures is quite reasonable. The SCOR-UNESCO results plotted against the PLS predicted value (Fig. 5.9) produces a plot that is not as good a regression as the previous

one. The plot of the SCOR-UNESCO values versus the HPLC values for chlorophyll 'a' (Fig. 5.10) indicates the SCOR-UNESCO equations to be underestimating the chlorophyll 'a' concentration.

Figure 5.11 shows the relationship for chlorophyll 'b' concentrations as determined using HPLC and the SCOR-UNESCO equations. One point of the test data lies well off the regression line. The PLS predictions for chlorophyll 'b' as determined using HPLC is not as good as for chlorophyll 'a'. Figure 5.12 is the graph of the chlorophyll 'b' concentrations determined using the SCOR-UNESCO equations versus the PLS predicted levels of chlorophyll 'b'. This graph like the previous shows a clustering of the data points. The test data however does lie closer to the regression line. Figure 5.13 is the plot of chlorophyll 'b' levels determined by HPLC against the chlorophyll 'b' levels determined using the SCOR-UNESCO equations. This plot shows that there is a clustering of the data points, with few of them anywhere near the regression line. The poor estimation of the chlorophyll 'b' concentrations may be attributed to the low detector response (Fig. 5.7) for chlorophyll 'b'.

Fig. 5.6. Detector Response for Chlorophyll 'a'

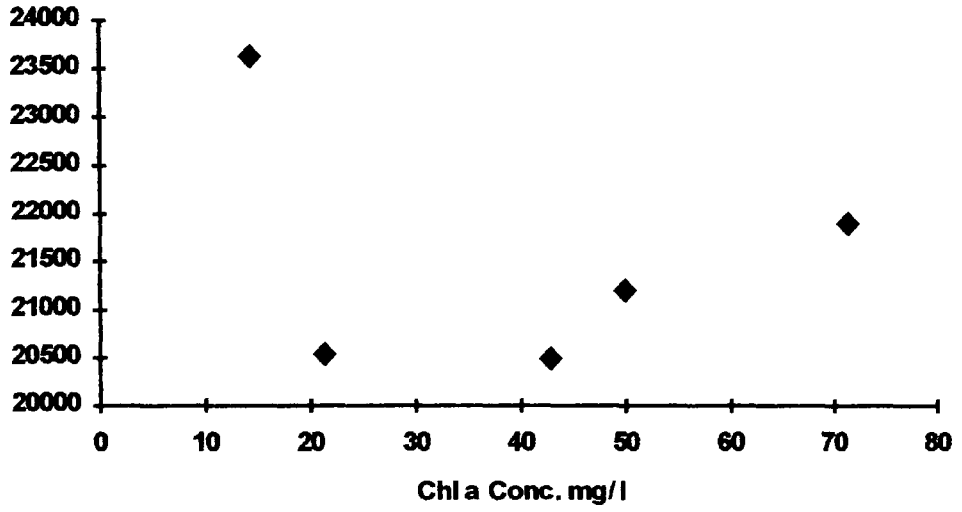


Fig. 5.7. Detector Response for Chlorophyll 'b'.

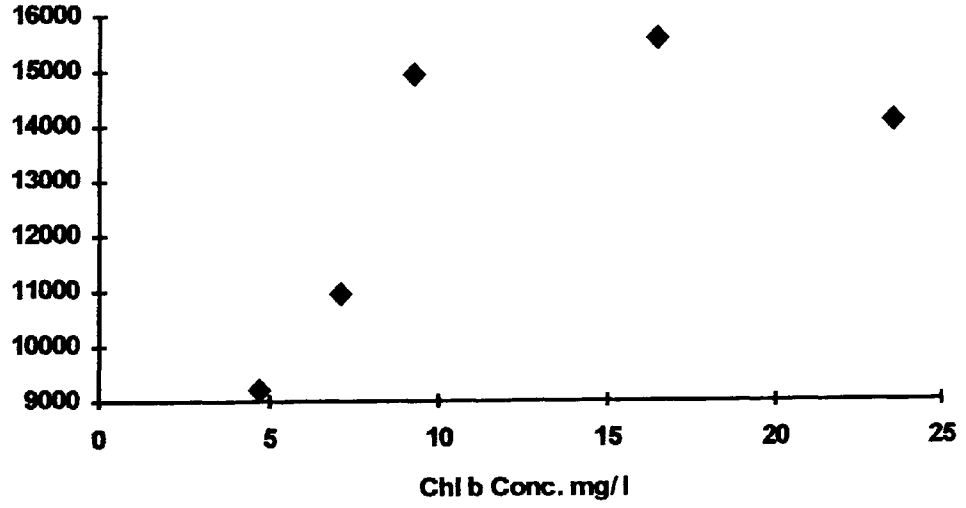
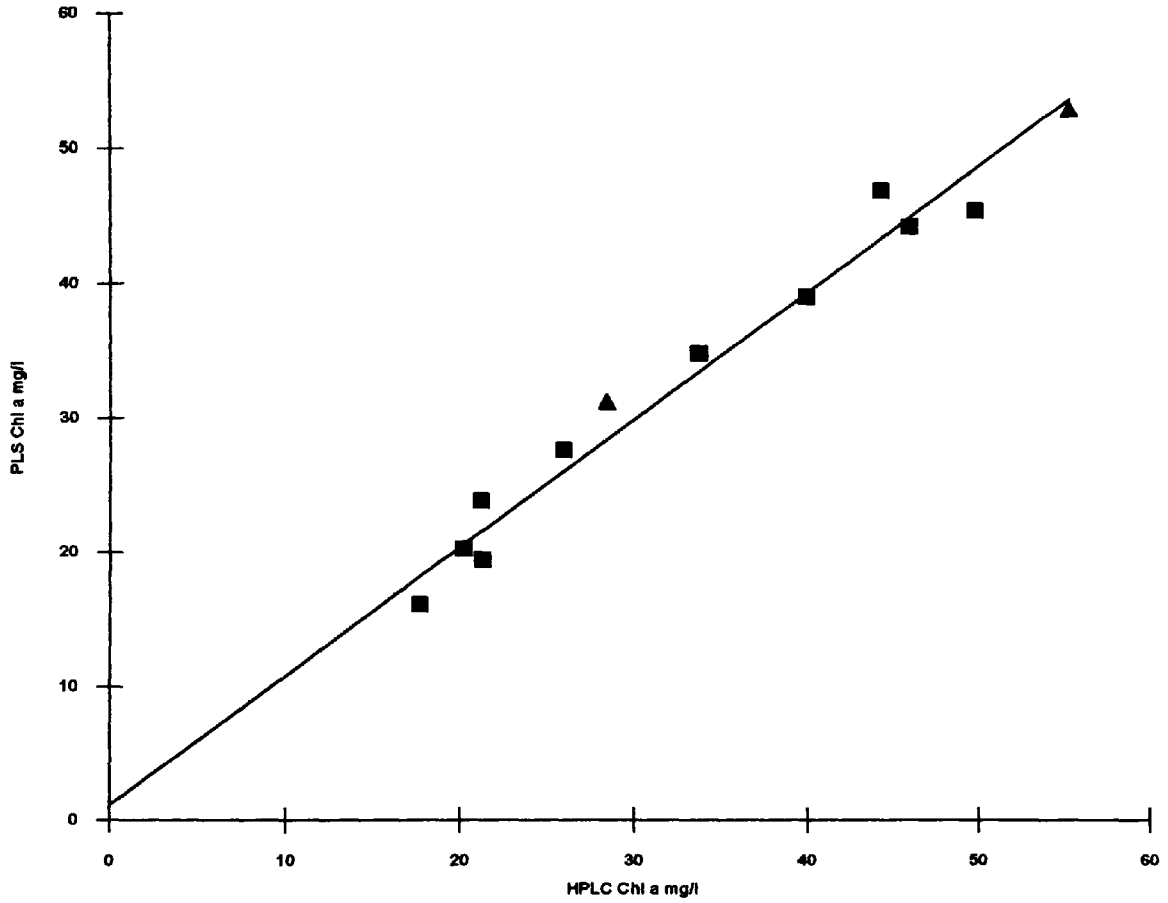
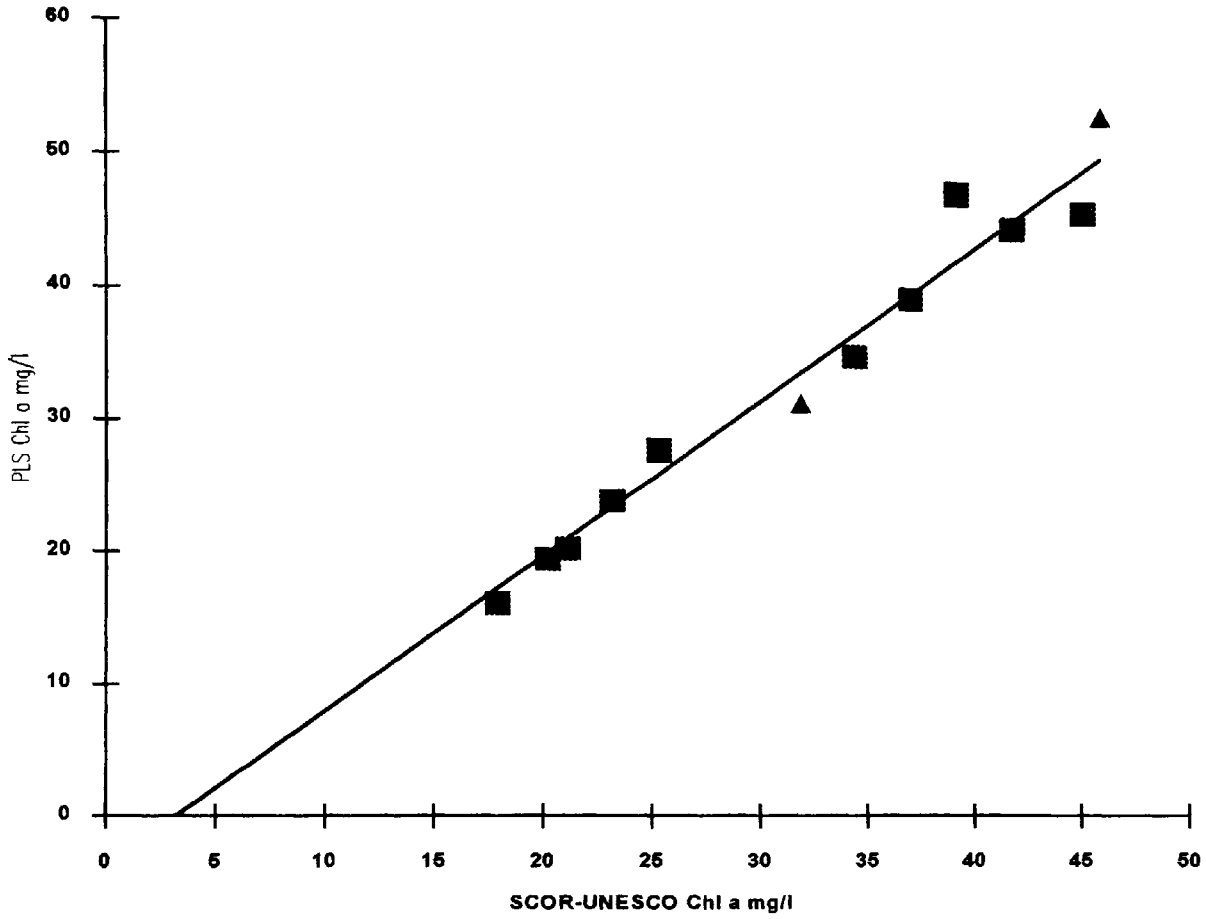


Fig. 5.8. HPLC Chlorophyll 'a' Levels versus PLS Predicted Chlorophyll 'a' Levels.



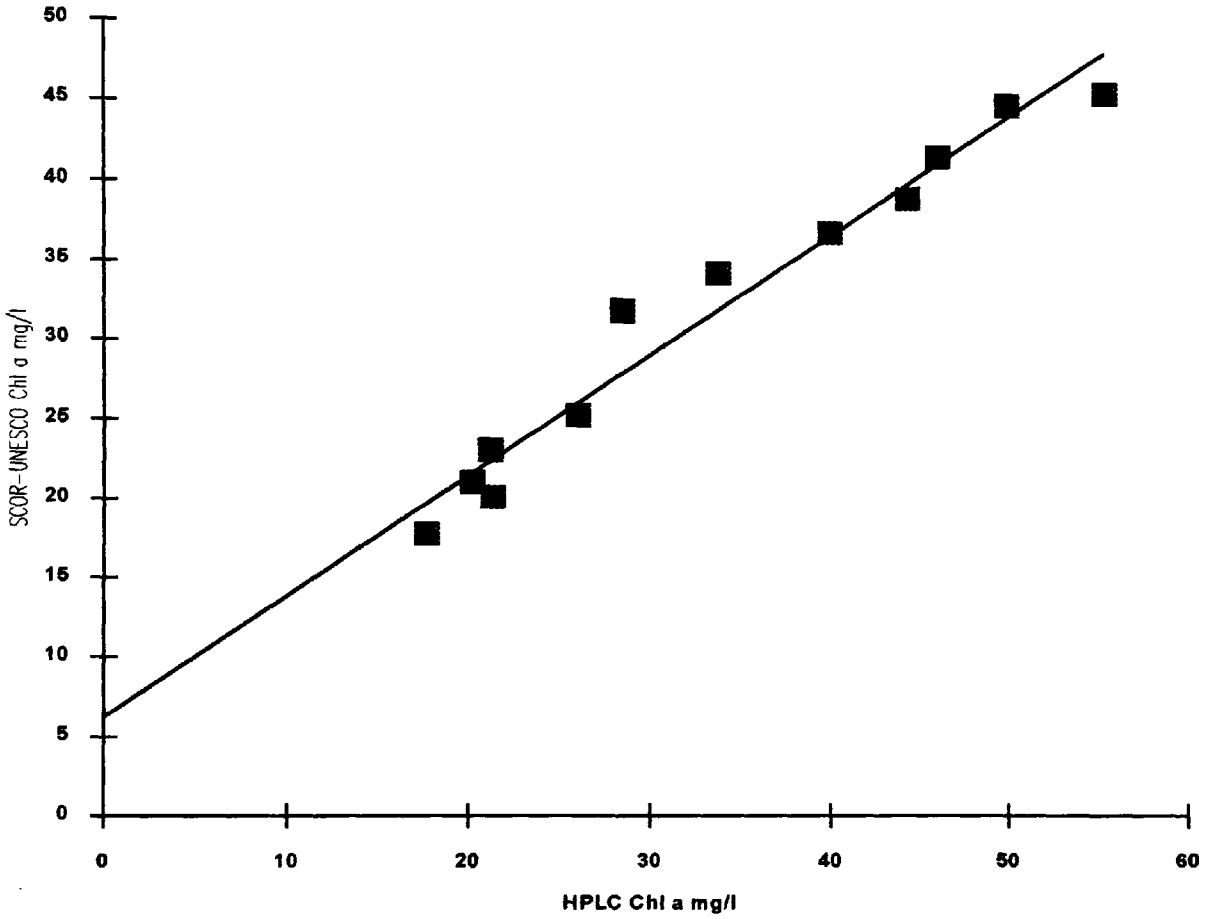
Gradient	0.962
Y Intercept	1.161
Correlation	0.984
Standard Error	2.279

Fig. 5.9. SCOR-UNESCO Chlorophyll 'a' Levels versus PLS Predicted Levels of Chlorophyll 'a'.



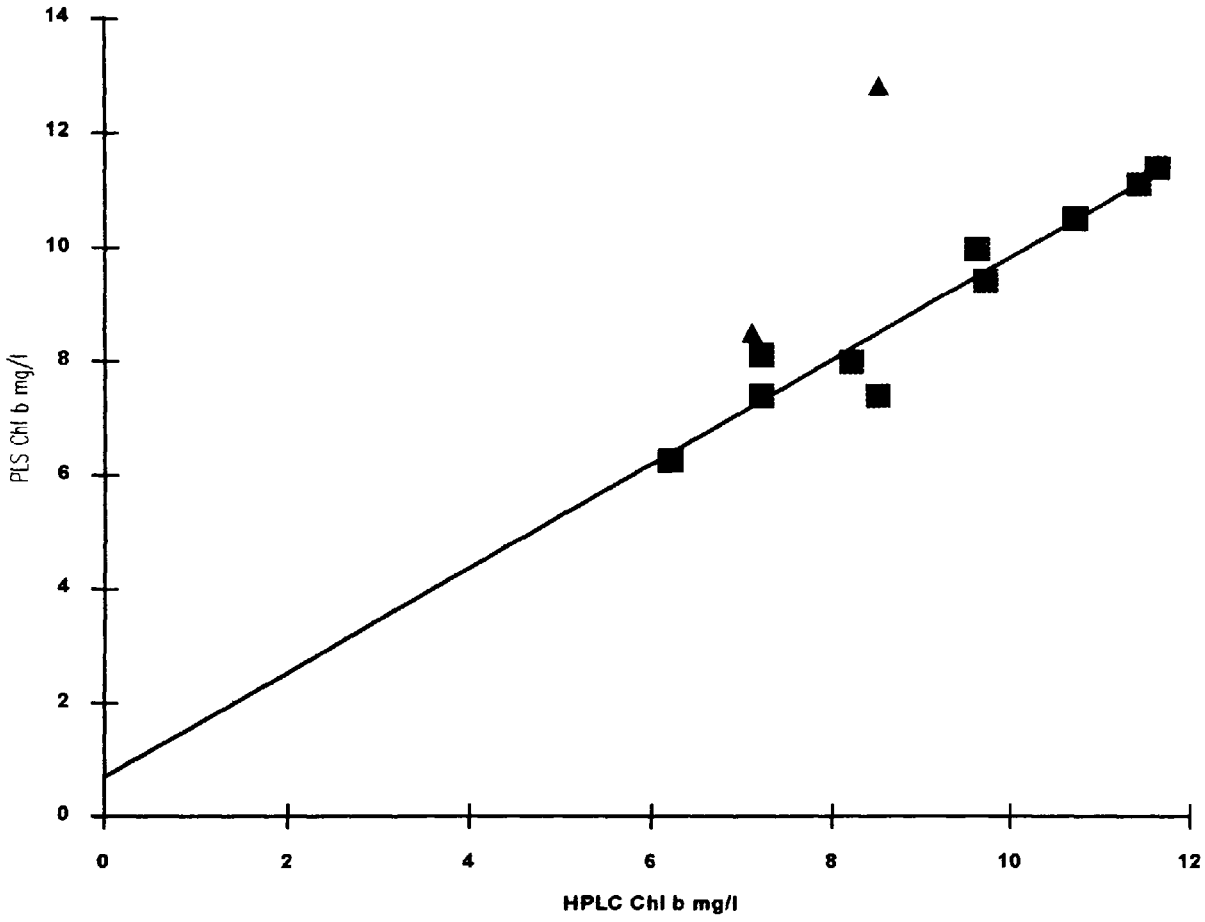
Gradient	1.173
Y Intercept	-3.733
Correlation	0.982
Standard Error	2.368

Fig. 5.10. HPLC Chlorophyll 'a' Levels versus SCOR-UNESCO Chlorophyll 'a' Levels.



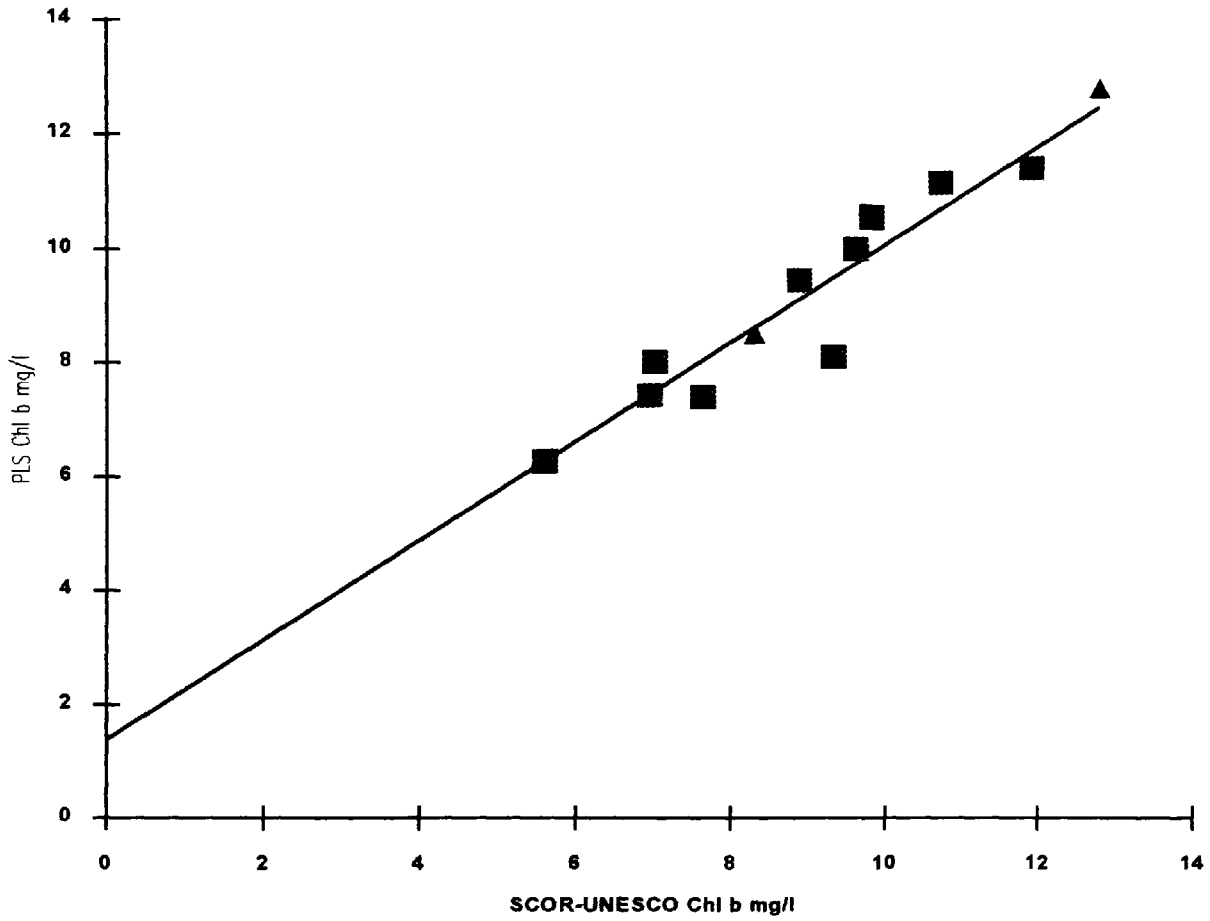
Gradient	0.760
Y Intercept	6.265
Correlation	0.921
Standard Error	1.982

Fig. 5.11. HPLC Chlorophyll 'b' Levels versus PLS Predicted Chlorophyll 'b' Levels.



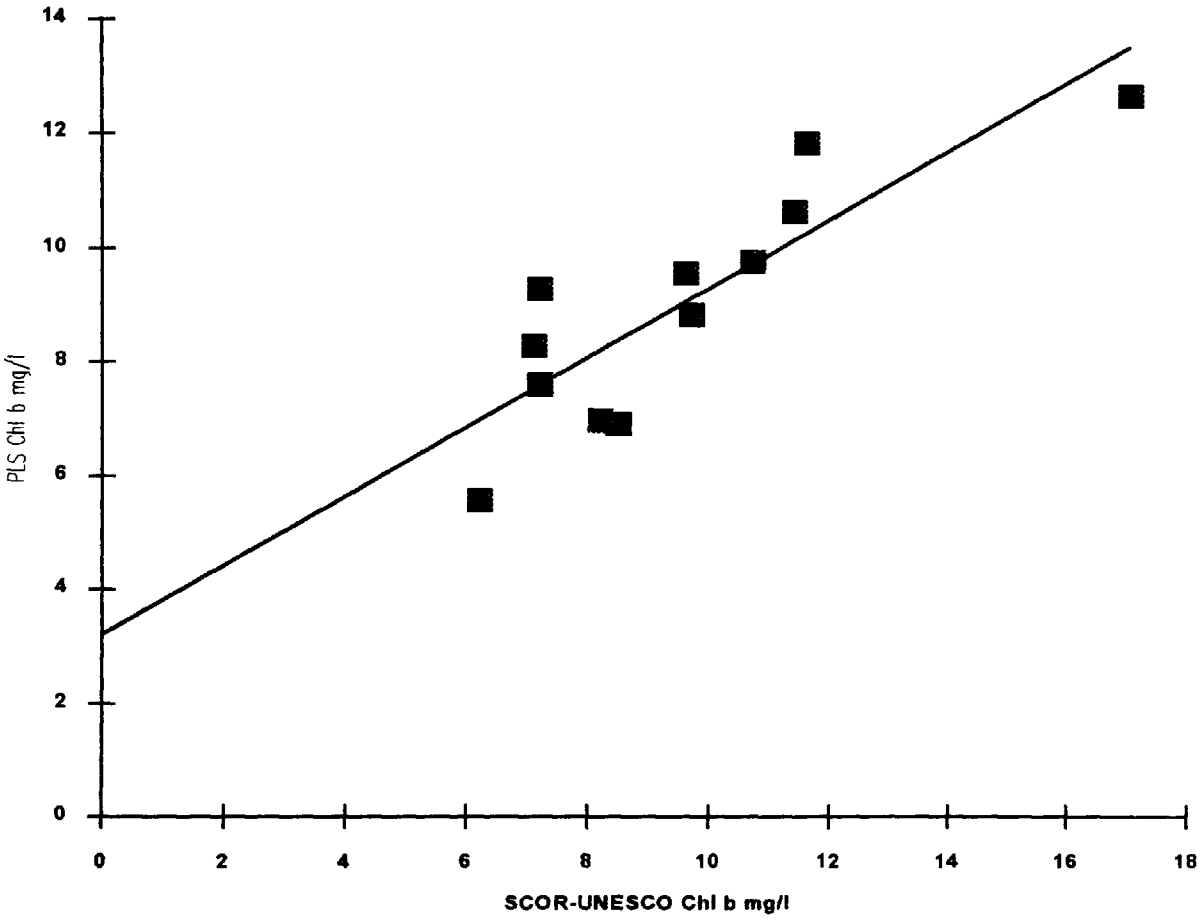
Gradient	0.923
Y Intercept	0.691
Correlation	0.923
Standard Error	0.527

Fig. 5.12. SCOR-UNESCO Chlorophyll 'b' Levels versus PLS Predicted Levels of Chlorophyll 'b'.



Gradient	0.875
Y Intercept	1.386
Correlation	0.938
Standard Error	0.661

Fig. 5.13. SCOR-UNESCO Chlorophyll 'b' Levels versus HPLC Levels of Chlorophyll 'b'.



Gradient	0.611
Y Intercept	3.202
Correlation	0.657
Standard Error	1.136

5.5 Development of a Procedure for the Separation and Analysis of Chlorophylls 'a' and 'b' Using Super Critical Fluid Chromatography.

The solvating power of supercritical fluids was demonstrated in 1895 by Hannay and Hogarth. They studied the solubility of cobalt and ferric chlorides in supercritical ethanol and found the concentrations of the metal chlorides to be much higher than the vapour pressures alone would predict [Peaden and Lee (1982)].

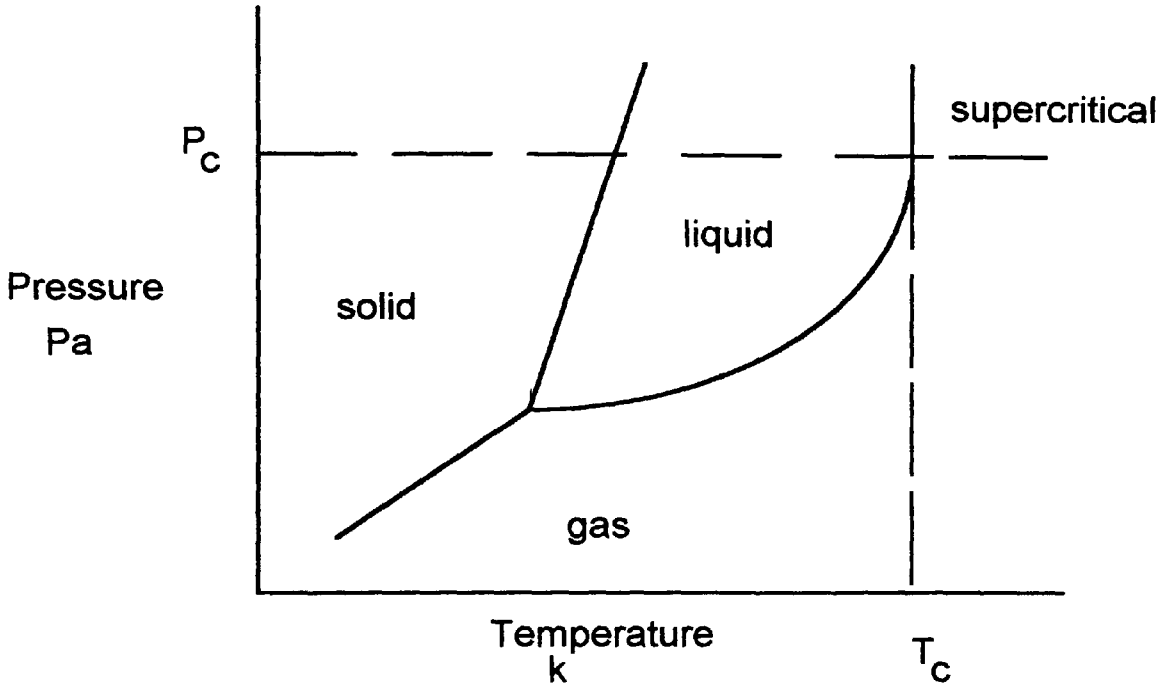
Supercritical fluid chromatography (SFC) was first reported by Klesper et al in 1962. Using various chlorofluoromethanes they demonstrated the separation of nickel porphyrin derivatives from each other. SFC has proved to be very useful in its ability to handle high molecular weight and thermally labile compounds. This has been shown by the types of compounds that have been separated using various supercritical mobile phases including alkyl bromides, aromatic hydrocarbons, polystyrenes, phospholipids, sugars, amino acids, various natural products, alcohols and acids [Peaden and Lee (1982)].

SFC, like other forms of chromatography, may be defined as a physical separation method based on the interaction of an analyte in a mobile phase with a stationary phase. With SFC though, the mobile phase is a substance that has been raised to a temperature and pressure where the liquid and vapour phases have the same density. At this transition point these are known as the critical pressure (P_C) and the critical temperature (T_C). Above P_C and T_C a low density fluid (gas-like) can be compared to a high density fluid (liquid-like) without gas to liquid condensation. Pressures typically used vary from about one-half to several times the critical pressure of the mobile phase [Peaden and Lee (1982)]. For carbon dioxide the T_C and P_C are shown in Fig. 5.14.

There are a number of features that make SFC very useful. Supercritical fluids have intermediate properties between those of gases and liquids [Peaden and Lee (1982), Klesper (1990)]. For example, the diffusion coefficients for solutes are higher in supercritical fluids than those in liquids but they are lower than in gases. Thus the optimum mobile phase flow rates are highest for gases, lowest for liquids, with supercritical liquids being between them [Peaden and Lee (1982) Lauer (1983)]. Thus the speed of analysis is expected to increase in the order; liquid chromatography, supercritical fluid chromatography and gas chromatography. [Peaden and Lee (1982)]. This intermediate diffusivity gives advantage to SFC over HPLC in that peak dispersion is decreased resulting in narrower peaks that aids in improving detection limits, faster analysis times for SFC that results in less peak broadening [Gere (1983)].

The viscosity of the mobile phase determines the pressure drop across a column that is required to obtain the desired flow rate. With gases and supercritical fluids the pressure drop in a column is similar. This is approximately a hundred times less than for HPLC [Peaden and Lee (1982)].

Fig. 5.14 Phase Diagram for Carbon Dioxide.



The viscosity of gases and supercritical fluids are very similar, whilst the viscosity's of liquids is about a hundred times greater than for gases and supercritical fluids. Thus with gases and supercritical fluids the pressure drop in a column is similar, but the drop in pressure when liquids are used, is among 10 to 100 times greater [Peaden and Lee (1982)].

By increasing the density of a supercritical fluid, the fluids' ability to dissolve higher molecular weight compound is increased [Peaden and Lee (1982)]. Thus, by using pressure control, the density of the supercritical fluid can be varied thus varying the solvating power of the mobile phase. So, by carefully increasing the density of the mobile phase during a chromatographic run, higher molecular weight compounds can be sequentially eluted [Peaden and Lee (1982), Klesper (1990)]. The molecular weight range that can be analysed by SFC in some cases extends over the full range of both gas and liquid chromatography.

There are a variety of mobile phases available for use in SFC. This adds the ability of mobile phase selectivity as a factor that can aid in separations of solutes [Peaden and Lee (1982)]. The diversity of mobile phases that are available for SFC is limited by the critical temperatures and thermal stability's of the fluids concerned. Table 5.6 gives the parameters for a number of common and potentially useful supercritical solvents [from Klesper (1990)].

Table 5.6. Potentially Useful Supercritical Solvents.

Fluid	Dipole Moment (debyes)	T_c (^{0}C)	P_c (atm)
CO_2	0.00	31.3	72.9
N_2O	0.17	36.5	72.5
NH_3	1.47	123.5	112.5
n-C_5	0.00	196.6	33.3
n-C_4	0.00	152.0	37.1
SF_6	0.00	45.5	37.1
Xe	0.00	16.6	58.4
CCL_2F_2	0.17	111.8	40.7
CHF_3	1.62	25.9	46.9

To exploit the chromatographic advantages of SFC, it is essential that the fluid mixtures used for the mobile phase be selected such that they can be mixed and pumped as a single phase, preferably at ambient temperature [Klesper (1990)]. Proper operating conditions must be chosen to give a single phase supercritical fluid, and care must be taken to avoid entering a two phase region when operating over a range of pressures as is typical of SFC.

The solvent strength of carbon dioxide varies considerably with the density of the fluid. The solvent strength at a density of 0.25 g cm^{-3} is less than that of the perfluorinated alkanes, while at a density of 0.98 g cm^{-3} it is greater than hexane [Gere (1983)]. It has been found that the solvent strength can be increased by adding miscible polar modifiers e.g. alcohols, ethers, tetrahydrofurans, diethylsulfoxide, chloroform etc. The use of carbon dioxide modifier mixtures offers a great deal of flexibility, as the modifier and its concentration are easily and widely variable and the solvent power can be altered to fir the analysis [Gere (1983)].

The selectivity and the maximum solvating power of the mixture are determined by the identity of the modifier, just as in HPLC [Gere (1983)]. It has been found that the amount of modifier affects the overall retention time as well as the elution order [Gere (1983)], Klesper (1990)].

The instrumentation of SFC resembles quite closely the equipment used in HPLC. This is due to both techniques using a solvent as the mobile phase together with high pressures. Where SFC differs in its instrumentation from that of HPLC is as follows:

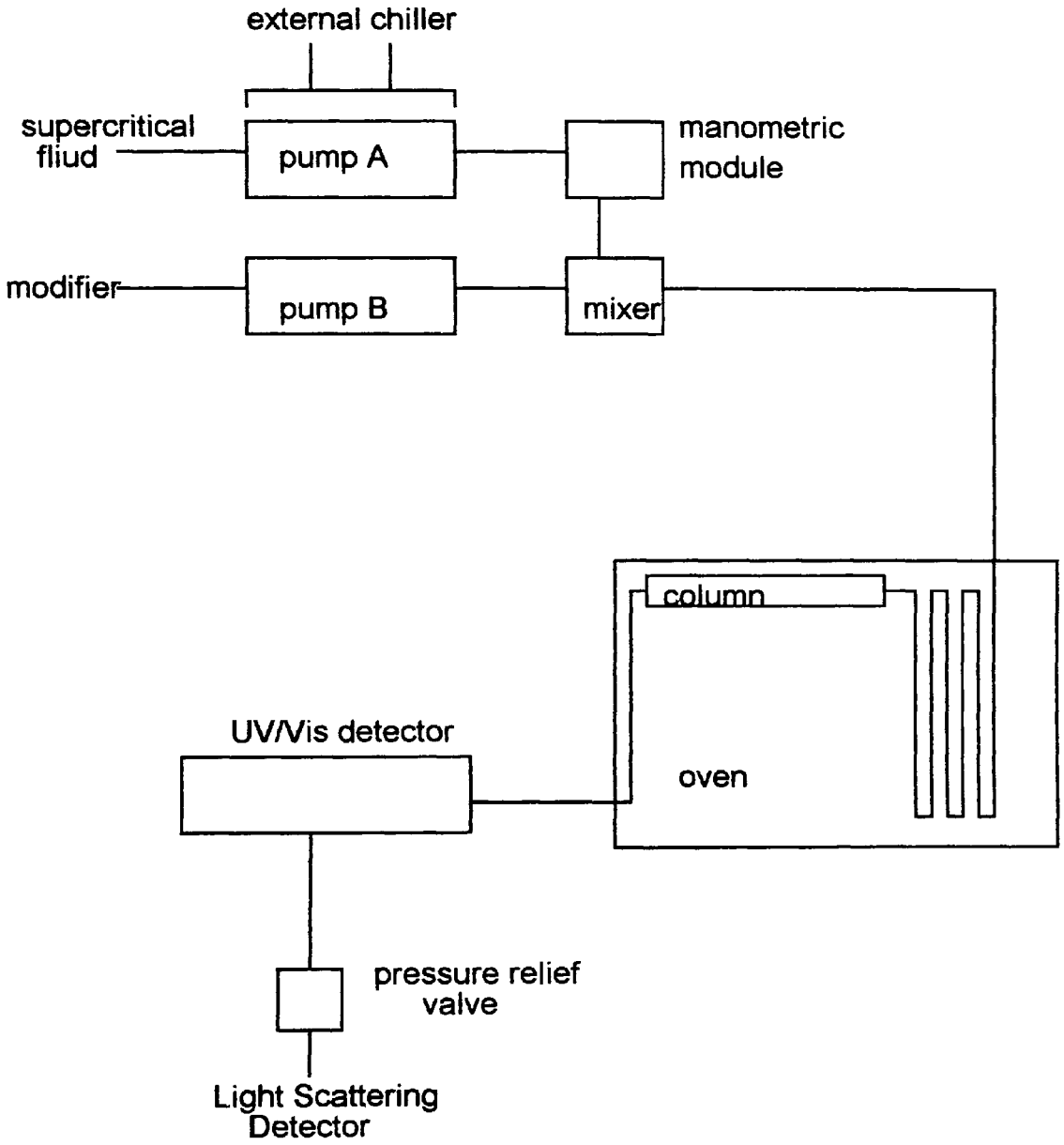
a) the entire column must be under high pressure

b) supercritical fluid chromatography requires pressure control rather than flow control

c) detectors must be designed to work under high pressures, or provide for a means to handle eluted compounds after the mobile phase pressure is reduced to atmospheric pressure. [Peaden and Lee (1982)].

This third point becomes important when the mobile phase used is normally a gas under atmospheric pressure and temperatures. When the mobile phase is allowed to come to atmospheric pressure, dissolved solutes will precipitate out. This has proved to be a major problem for mass spectrometer interfaces and flame ionisation detectors [Peaden and Lee (1982)]. A basic outline for the instrumentation involved in a SFC set up is shown in Fig. 5.15.

Fig. 5.15. Diagram of a Typical SFC System.



The types of solvent delivery system have differed in the main, with respect to the mobile phase that is going to be used. With mobile phases that are gases at ambient pressure, a gas cylinder has been connected to high pressure reducing valves in order to supply the necessary mobile phase pressure to the column [Peaden and Lee (1982)].

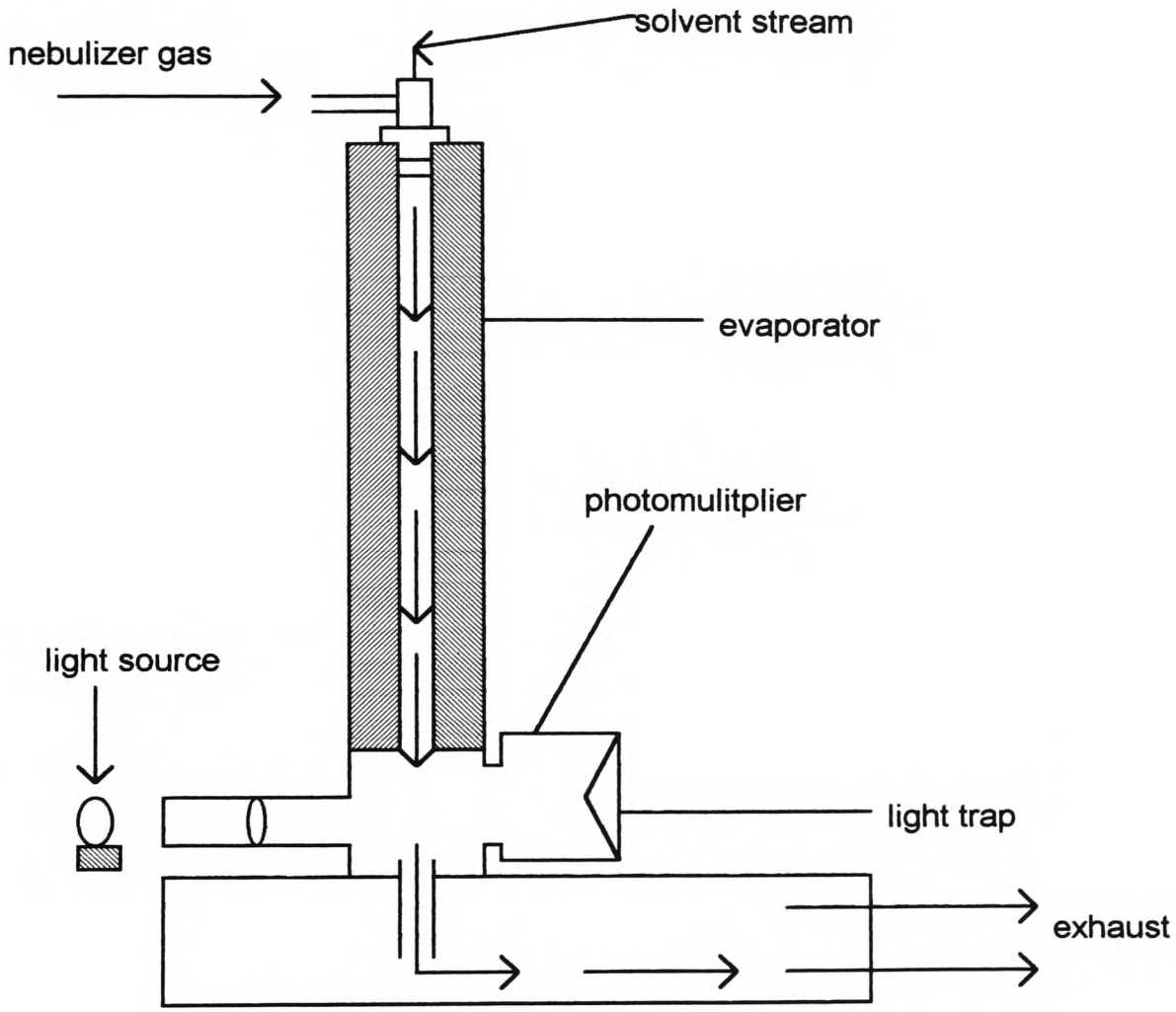
With SFC if higher pressures are required than those that can be supplied by the cylinder, there are two methods can be used to achieve the necessary pressure. One method involves the heating of the cylinder and another the pressure to the column has been raised via the use of high pressure pumps [Peaden and Lee (1982), Klesper (1990)].

For mobile phases that are liquids at ambient conditions, high pressure liquid chromatographic pumps can be used after they are modified for pressure control, rather than for flow control [Peaden and Lee (1982)]. It is necessary that the flow output from the pumps should be pulseless. Reciprocating piston pumps require pulse damping systems to ensure pulseless operation. Syringe pumps on the other hand, can deliver a pulseless flow without damping. The pressure control system should also allow for pressure programming in order to increase its effectiveness [Peaden and Lee (1982)].

The injection systems that are used in SFC are similar to those used in HPLC. Various kinds of high pressure valves have been used in SFC as part of the injection system . It is advantageous if the system allows the supercritical fluid mobile phase to be the injection solvent. Care has to be taken to ensure that the sample is soluble in the mobile phase under the injection conditions [Peaden and Lee (1982)]. The temperature of the column and of the mobile phase is controlled by some form of heating oven. In the oven the column and a coil for preheating the mobile phase are situated. Maintaining accurate temperature control becomes of paramount importance when one is worker close to or at the critical temperature of the mobile phase [Peaden and Lee (1982)].

The most popular form of detector used in SFC is the UV/Visible detector. Modifications have to be made to the instrument to meet the needs of SFC. Capillary columns require on-column detectors to meet their small volume requirements. The detectors employed in HPLC are not designed to operate at the high pressures used in SFC. To be employed successfully in SFC they have to be fitted with a high pressure cell to detect solutes in the supercritical mobile phase [Peaden and Lee (1982)]. Systems have been devised to allow for cooling of the mobile phase to the liquid state, followed by detection in low pressure UV detector cells [Peaden and Lee (1982)]. Flame ionisation detectors have been successfully used, but those do tend to present some problems because of sample condensation during decompression of the mobile phase [Peaden and Lee (1982)]. This problem is also present in the interfacing of supercritical fluid chromatographs to mass spectrometers. Other types of detectors used include refractive index, adsorption, scintillation and thermoconductivity detectors [Peaden and Lee (1982)]. A more recent type of detector to be successfully employed for SFC is the light scattering detector. This device works on the basis of light scattering, the light being scattered by the particles contained in the solvent. The solvent is evaporated in the device and the solute particles scatter light that is detected via a photo multiplier tube. The apparatus is shown in Fig. 5.16.

Fig. 5.16. Diagram of a Light Scattering Detector.



5.5.1. SFC Method and Instrumentation.

The set up consisted of two Gilson 303 pumps, one being adapted to pump the gas, a Gilson 802C manometric module for monitoring the pressure of the system, a Gilson 811 dynamic mixer for mixing the modifier with the supercritical fluid and an oven (Anachem) for heating the column and coil for the supercritical fluid. The system was linked to a Kratos Spectraflow 757 UV/visible detector and a Pye Unicam DP 88 computing integrator. An Apple IIe micro computer with Gilson software was used for programming modifier gradients and flow rates. Pressure was regulated using a back pressure regulator situated after the flow through cell. The inlet valve was fitted with a 20 μl injection loop.

5.5.2. Experimental and Results.

Chlorophylls 'a' and 'b' were obtained as previously described in this chapter. A method was also used to obtain the chlorophylls 'a' and 'b' using a preparative HPLC method. This was performed on a Varian Vista 5000 LC using a preparative Varian Micropak C-18 reversed phase column (300mm x 8mm), using an isocratic system of 100% acetonitrile. This method proved not to be as practicable in the TLC method. A sugar column was used to obtain chlorophylls 'a' and 'b'. The column was made up using icing sugar (Silver Spoon) and the eluent was 0.5% Isopropyl alcohol in petroleum ether (40-60).

Initially the work was carried out using a Waters micro bondapak C-18 reversed phase column. The conditions used were an oven temperature of 85^{o}C. The modifier was set on a gradient from 6% to 12% over a period of 8 minutes. The pressure was 4750 psi at a flow rate of 5 cm^3 min^{-1}. The supercritical fluid used was carbon dioxide.

The chromatogram is shown in Fig. 5.17. The separation of the chlorophyll mixture was not baseline. This leads to the conclusion that C-18 reversed phase may not be able to separate chlorophylls via supercritical chromatography, although the use of other super critical solvents may prove otherwise.

The next column type used was an amine Alphasil 5μm (250mm x 4.6mm). Initial results using carbon dioxide as the supercritical solvent and methoxyethanol were poor. It appeared that chlorophyll 'b' was being retained on the column. This could be due to the extra carbonyl group that is present on chlorophyll 'b' (Fig. 5.18) forming an imine with the NH_2 groups of the amine column.

By using methanal (40% aqueous solution) mixed with the modifier in the ratio 97% methoxyethanol/3% methanal solution, a successful separation of a mixture of chlorophyll 'a' and chlorophyll 'b' was achieved (Fig. 5.19). It is possible methanal was actively modifying the column during the chromatographic run. Since methanal has no asymmetric carbon centre it could form the imine with the $-NH_2$ of the packing material in the column. The experimental conditions were 80% carbon dioxide/20% modifier (3% methanal/97% methoxyethanol), the detector was set at 436ηm , oven temperature was 130 ^{o}C, pressure 3500 psi and a flow rate of 5 cm^3 min^{-1}

$$H_2C=O + H_2NR \longrightarrow HOCNHR \longrightarrow C=NR$$

Primary amine Imine

The imino form is the more stable, so if any enamine forms, the enamine quickly undergoes tautomerism to form the imine. This is analogous to keto-eno tautomerism. In the intermediate compound the proton is acidic and therefore quite readily separates to form the hybrid anion and it can return to either the carbon or the nitrogen. When it returns to the carbon it tends to stay there. The equilibrium tends to favour the weaker acid [Morrison and Boyd (1973)].

The minimum detection limits were determined by serial dilution. The minimum detection limit of chlorophyll 'a was found to be an injection containing 1.8ηg, whilst for chlorophyll 'b' the minimum detection limit was found to be 2.01 ηg. Both had a signal to noise ratio of 3:1. The injections were made from a solution of chlorophyll 'a' that was a 1/100 dilution of the stock solution (18.1 mg dm^{-3}) and a 3/100 chlorophyll 'b' stock solution (6.7 mg dm^{-3}). The chromatogram for the detection limit of chlorophyll 'a' is Fig 5.20 and for chlorophyll 'b,' Fig 5.21.

Fig. 5.17. Chromatogram of the Separation of a Mixture of Chlorophyll 'a' and 'b', Using a Waters Microbondapak C-18 Reversed Phase Column (5mm x 250mm). Oven temperature was set at 85 ^{0}C, pressure was 4750psi with a flow rate of 5cm^{3} min^{-1}. Modifier was set on a gradient of 6% to 12% over 8 minutes. The detector was set at 436ηm with a range of 0 to 0.05 a.u.f.s.

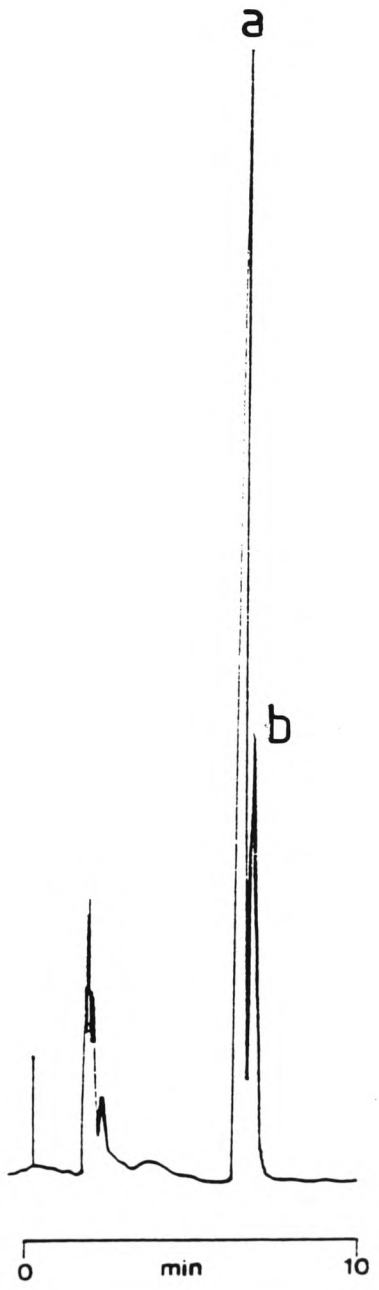
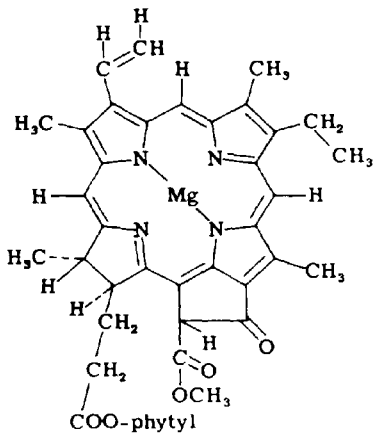
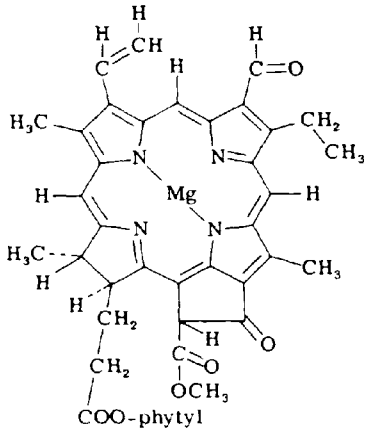


Fig. 5.18. Structure of Chlorophylls 'a' and 'b'.



Chlorophyll *a*



Chlorophyll *b*

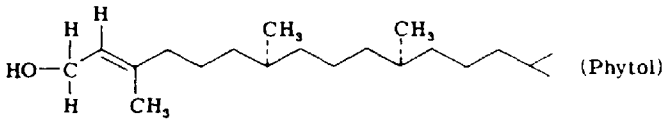


Fig. 5.19. Chromatogram Showing the Separation of a Mixture of Chlorophyll 'a' and 'b' using an Amine Alphasil 5μm (250mm x 4.6mm) Column. The modifier was 97% methoxymethanol/3% methanal. Flow rate was 5cm^3 min^{-1} using 80% CO_2/20 modifier. Oven temp. was 130 ^{0}C, pressure was 3500psi, detector was set at 436ηm with a range of 0 to 0.05 a.u.f.s.

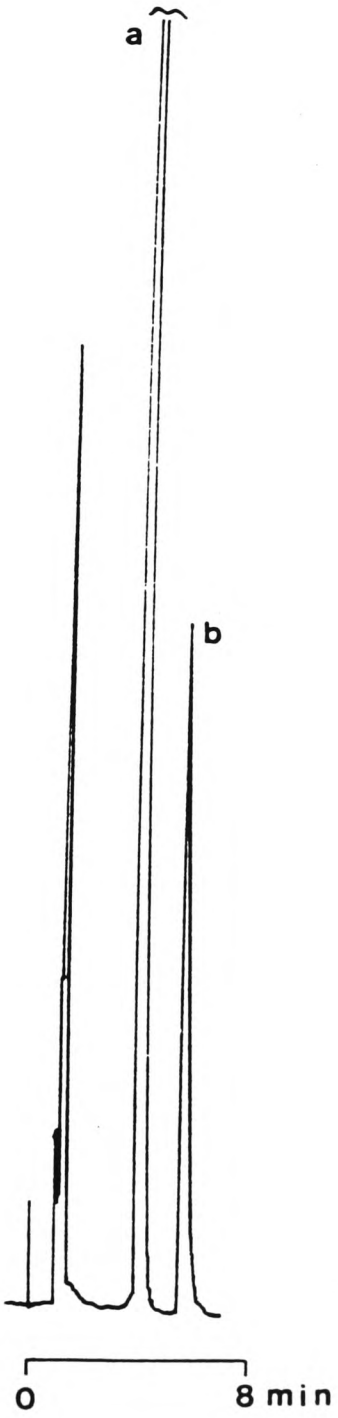


Fig. 5.20. Detection Limits of Chlorophyll 'a' Amine Alphasil 5μm (250mm x 4.6 mm) Column. The modifier was 97% methoxymethanol/3% methanal. Flow rate was 5 cm^3 min^{-1} using 80% CO_2/20 modifier. Oven temp. was 130 ^oC, pressure was 3500psi, detector was set at 436ηm wit a range of 0 to 0.005 a.u.f.s.

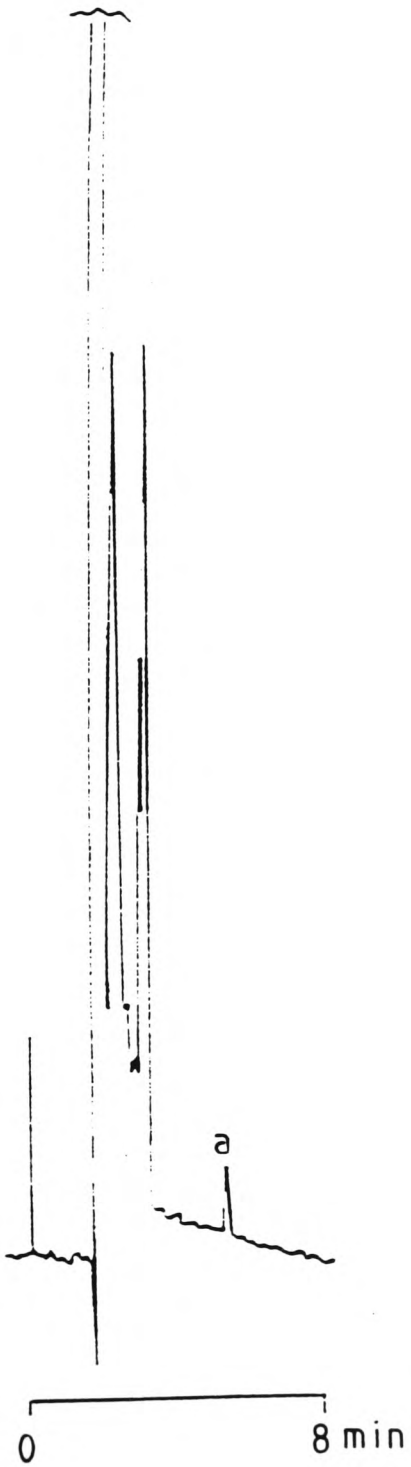
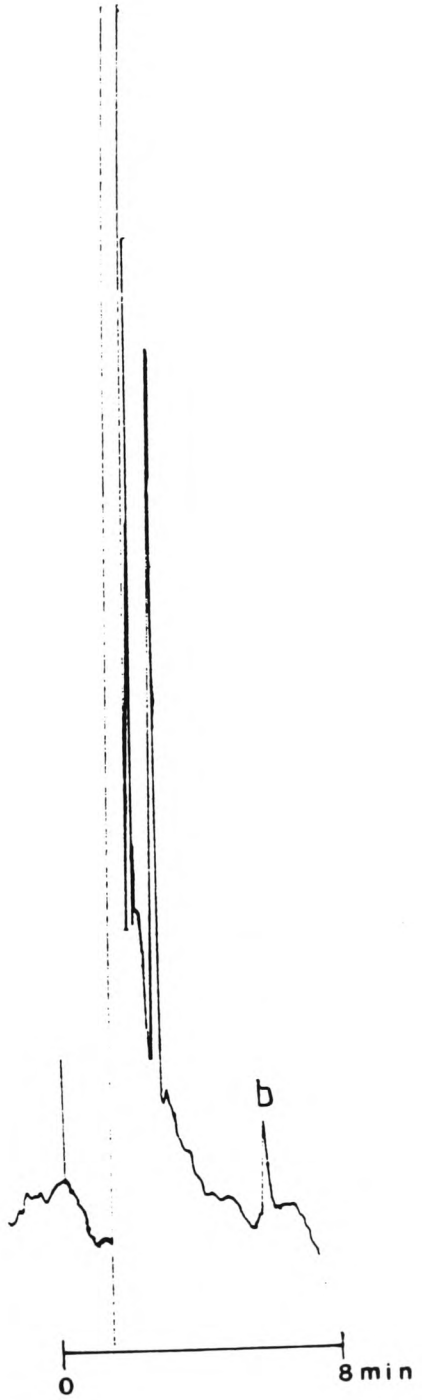


Fig. 5.21 Detection Limits of Chlorophyll 'b'. Amine Alphasil 5μm (250mm x 4.6 mm) Column. The modifier was 97% methoxymethanol/3% methanal. Flow rate was 5 cm^3 min^{-1} using 80% CO_2/20 modifier. Oven temp. was 130 ^0C, pressure was 3500psi, detector was set at 436ηm with a range of 0 to 0.005 a.u.f.s.



Under the experimental conditions the reproducibility of the method was determined using a mixture of chlorophyll 'a' and chlorophyll 'b' of concentrations 80.51 mg dm^{-3} and 20.4 mg dm^{-3} respectively. 20 µl injections were used. The results are given in Table 5.7. The ratios of the peaks were calculated and their consistency used to check reproducibility.

Table 5.7. Peak Areas and Ratios of Chlorophylls 'a' and 'b'

Run	Peak Area Chl 'a'	Peak Area Chl 'b'	a/b Ratio	b/a Ratio
1	47418	11184	4.249	0.235
2	46825	10903	4.295	0.233
3	48329	12650	3.820	0.262
4	47771	11547	4.137	0.244
5	47378	11357	4.172	0.240
6	49052	12204	4.019	0.241
7	47829	11527	4.149	0.241
8	50859	11591	4.388	0.228
9	50355	11601	4.341	0.230
10	51138	11736	4.357	0.229
Mean	48705.4	11630	4.193	0.239
sd	1559.7	494.7	0.175	0.011
%sd	3.2	4.3	4.2	4.6

The overall reproducibility is quite reasonable with the percent standard deviations of around 4.

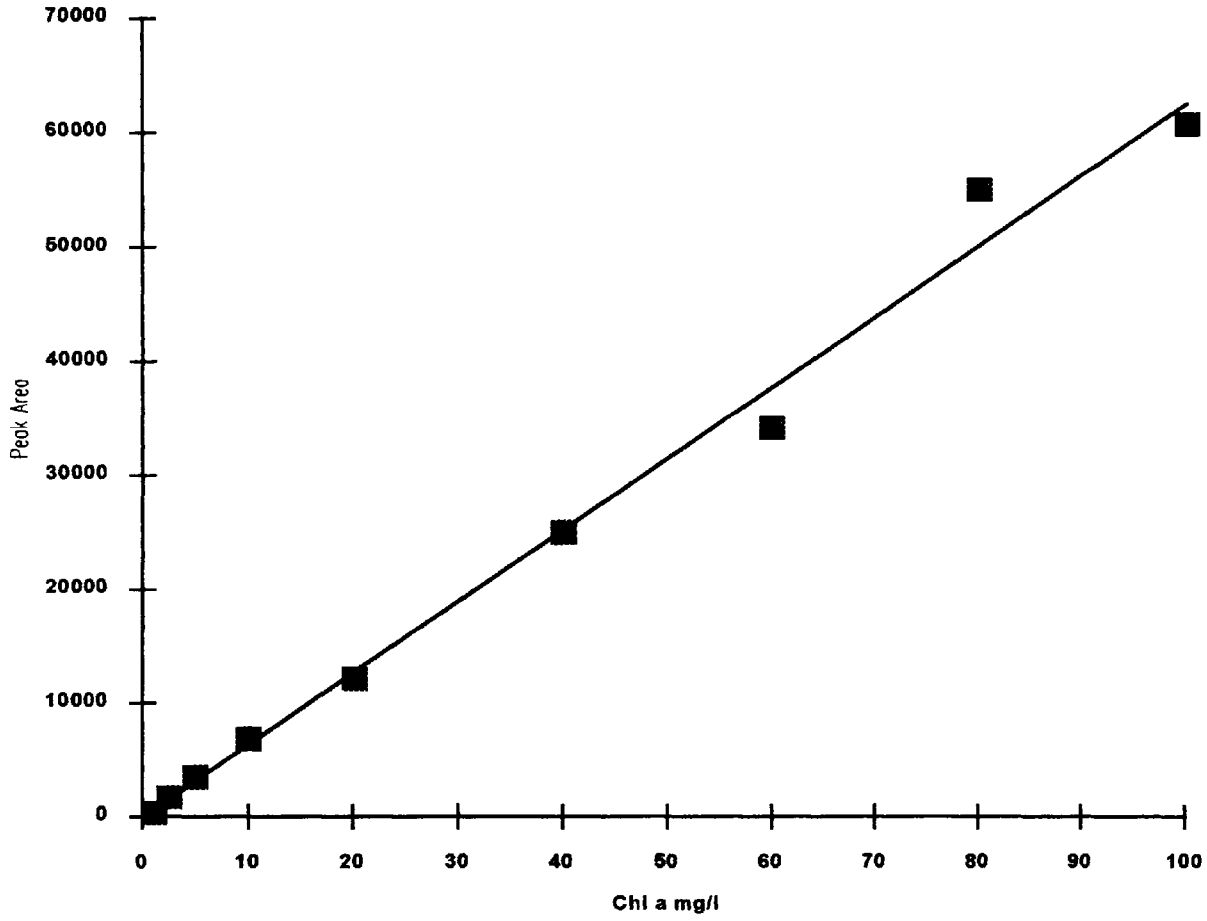
The method was then calibrated using dilution's of standard chlorophyll 'a' and chlorophyll 'b'. The chlorophylls were obtained from Sigma, Poole, Dorset, UK. The results of the mean of peak areas of four injections together with their respective standard deviations are given in Table 5.8. The respective calibration plots are given in Figs. 5.22 and 5.23.

Table 5.8. Results for the Calibrations of Chlorophylls 'a' and 'b'.
Chlorophyll 'a' Chlorophyll 'b'

Conc mg dm^{-3}	Mean Peak Area	sd	%sd	Mean Peak Area	sd	%sd
1	436.00	113.71	26.08	773.50	185.56	23.99
2.5	1831.25	240.72	13.14	1004.17	150.34	14.97
5	3631.25	206.23	5.68	3269.17	530.41	16.22
10	6886.75	339.80	4.93	6101.17	723.82	11.86
20	12234.50	495.09	4.05	11913.63	538.21	4.52
40	25134.00	979.85	3.90	21719.50	531.31	2.45
60	34466.00	1046.68	3.03	30970.00	1013.03	3.27
80	55566.75	2531.40	4.56	48053.13	1083.97	2.26
100	61367.50	581.23	0.01	57440.63	1421.84	2.48

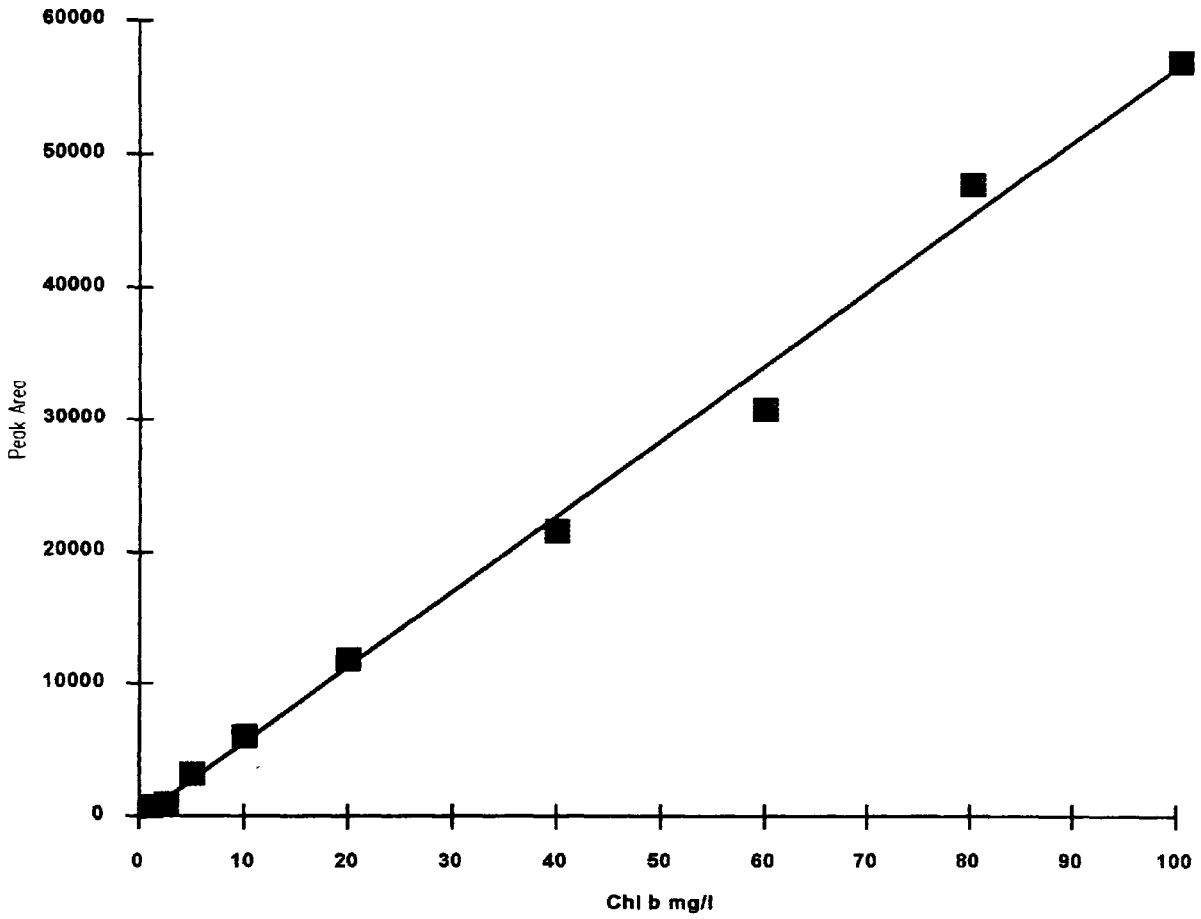
The detector response for chlorophylls 'a' and 'b' were checked and the results are shown in Figs. 5.24 and 5.25. These are plots of the average concentrations used in the calibrations against the average peak area divided by the chlorophyll concentrations. The response graphs show a reasonable degree of linearity for chlorophyll 'a' and 'b'. With both chlorophylls there is a loss of linearity at lower concentrations.

Fig. 5.22. Calibration Graph for Chlorophyll 'a'.



Gradient	0.995
Y Intercept	44.478
Correlation	0.995
Standard Error	2425.36

Fig 5.23. Calibration Graph for Chlorophyll 'b'.



Gradient	572.711
Y Intercept	-129.288
Correlation	0.997
Standard Error	1627.51

Fig. 5.24. Detector Response for Chlorophyll 'a'.

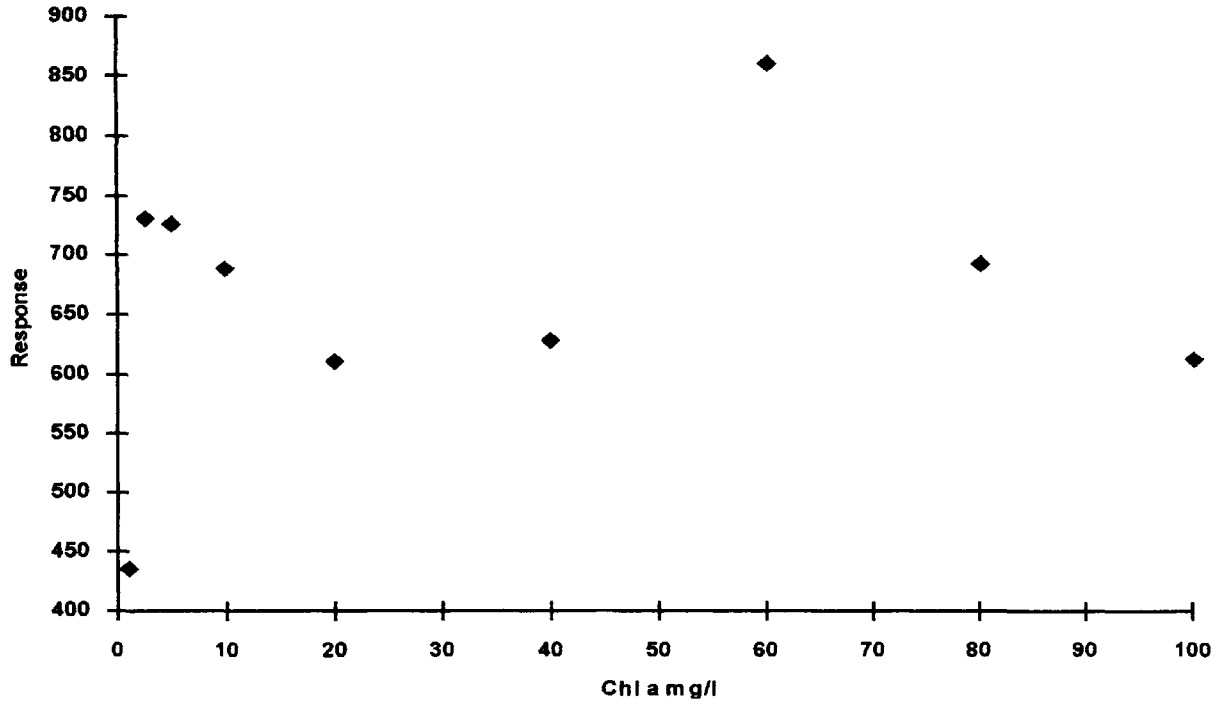
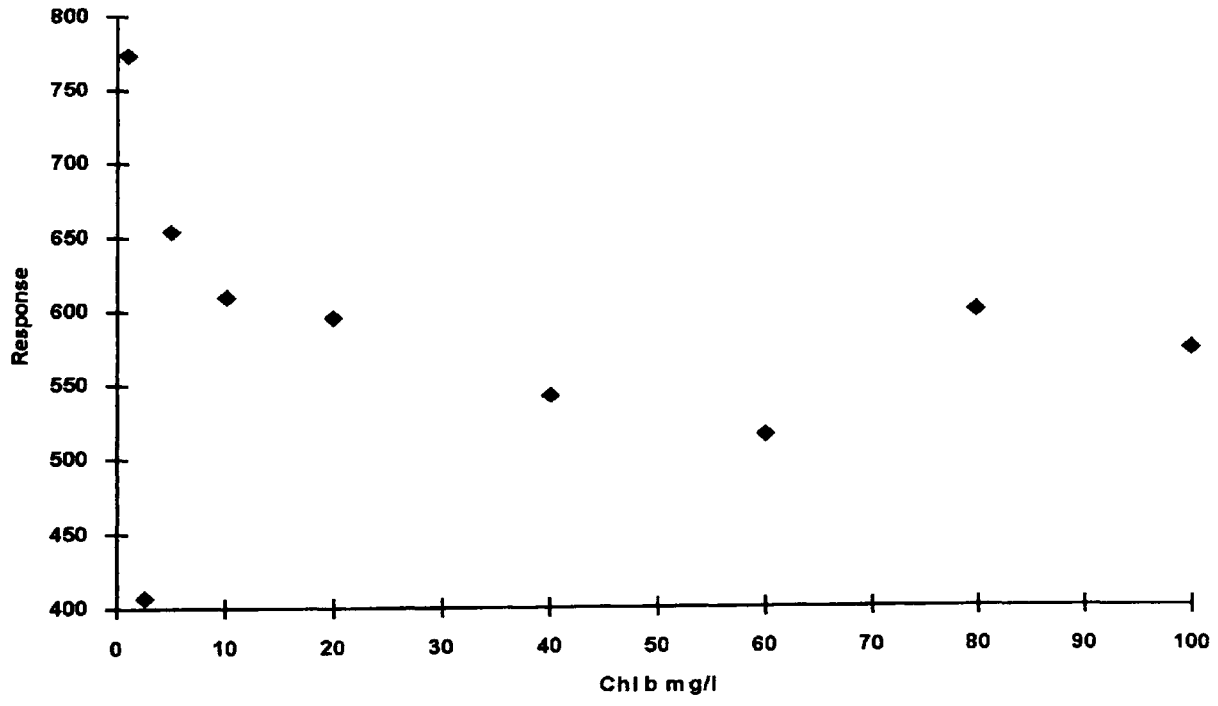


Fig. 5.25. Detector Response for Chlorophyll 'b'.



The method was also used with a light scattering detector. The chromatogram is shown in Fig. 5.26. The experimental details were the same as for the previous chlorophyll separations using the amine column.

The method for separating a mixture of chlorophylls 'a' and 'b' using packed column SFC appears to be quite promising. It out scores the HPLC method in its speed of analysis. The run time using the SFC is around six to seven minutes whereas that of the HPLC for the separation of a mixture of chlorophyll 'a' and 'b' is about twelve minutes, which is double that of the SFC method.

The SFC method was then applied to a plant pigment extraction. The chromatogram of which is shown in Fig. 5.27. The result indicates a good separation of the two chlorophylls from each other, but non-baseline separation between the chlorophyll 'a' and the carotenoids.

Fig. 5.26. Separation of a Mixture of Chlorophyll 'a' and 'b' with Detection Using a Light Scattering Detector. The column used was an Amine Alphasil 5μm (250mm x 4.6mm) using 97% methoxymethanol/3% methanal as a modifier. The flow rate was 5 cm^3 min^{-1} using 80% CO_2/20% modifier. Oven temp. was 130 ^oC, pressure was 3500psi, detector was set at 436ηm with a range of 0 to 0.05 a.u.f.s.

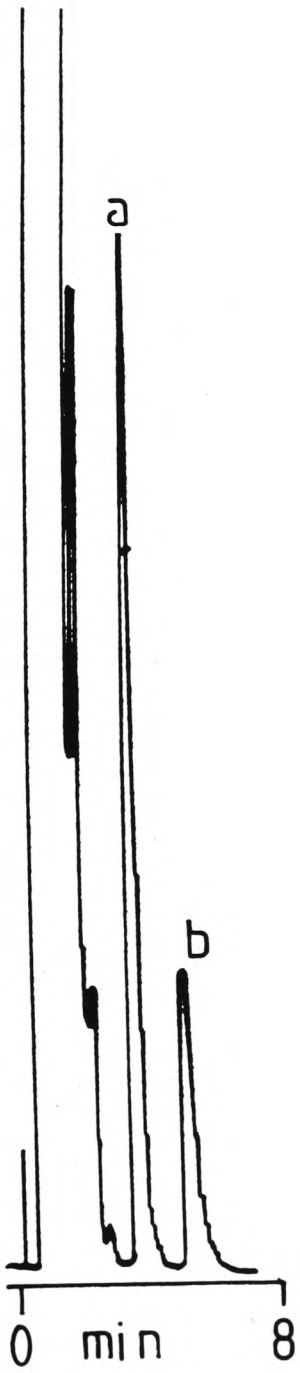
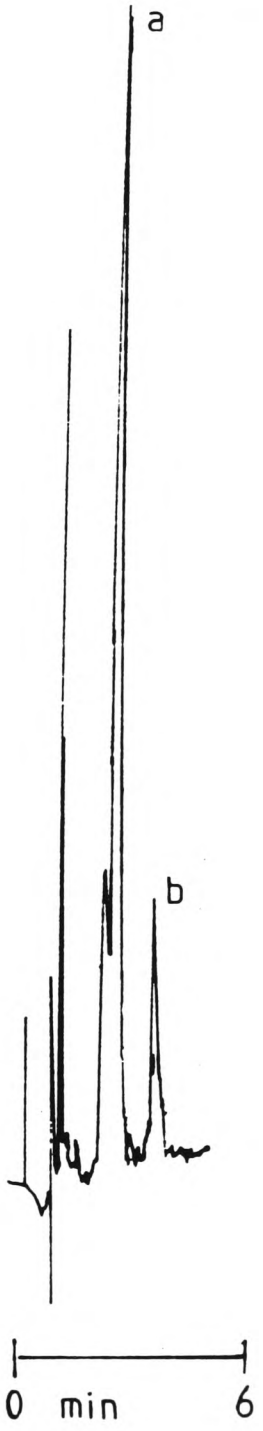


Fig. 5.27. Chromatogram of a Plant Pigment Extract. Amine Alphasil 5μm (250 mm x 4.6mm) Column. The modifier was 97% methoxymethanol/3% methanal. Flow rate was 5 cm^3 min^{-1} using 80% CO_2/20 modifier. Oven temp. was 130 ^0C, pressure was 3500psi, detector was set at 436ηm with a range of 0 to 0.05 a.u.f.s.



The method must be further developed so that it can be used in the routine analysis of chlorophylls in plant extracts. Analytical SFC is still in its infancy and the method is still being developed. Recently supercritical fluid extraction (SFE) is now becoming popular. This method may revolutionise the extraction of materials from various matrices. SFE allows the density of the supercritical fluid to be varied (as with SFC) and this in turn allows compounds of various molecular weights and/or polarities to be extracted from a medium. This method could be developed for the selective extraction of plant pigments from leaves and needles together with the on-line analysis of the pigments...so there is plenty of scope for the further development of this technique for this type of analysis! Other packing materials may prove more suitable for the analysis of plant pigments together with the use of other super critical mobile phases and modifiers. Possibly the use of a C-18 in conjunction with an amine column may be suitable.

CHAPTER 6

6. Interactive Effects of Heavy Metals on Two Year Old Sitka spruce.

The initial survey work carried out at the Afan 1 forest sites, brought to light significant differences in the K levels between the good growth sites and the poor growth sites for the year 1, year 2 and year 3 needles. A similar result was found for Ca in the year 1 and year 2 needles. A deficiency in any one of these two nutrients could lead to stunted growth and needle loss [Mengel and Kirkby (1982)]. The differences in the levels for the two nutrients may be due to any number of factors or combinations of these. Other elements in the soil for example may affect the uptake of these nutrients. However, although survey work may indicate such relationships, it is only through experimentation that cause and effect may be determined.

Cadmium is a well-known toxic metal. From the survey work (section 2.3) it was apparent that trees at some of the sites had foliar concentrations of cadmium above the background levels. Cadmium has been shown to affect root development in tree seedlings [Malone et al (1977), Godbold et al (1985)]. This metal in various combinations with another element such as Cu (which in excess is also toxic to plants) [Morgan (1983)] could be used in an experiment to determine their effects upon the growth and various nutrient levels in young Sitka spruce trees. Also cadmium and copper in combination have been shown to affect the uptake of K in cereal roots [Bujtas and Cseh (1981)].

Any experiment investigating such effects requires seedlings or young trees because of the length of time for any effects to become apparent. However, a number of problems

may arise with the use of such young trees or seedlings. These include the difference in age between the trees used and the age of the trees found at the Afan 1 forest. Often the time span of the experiment and the concentrations of the toxic metals is important. If the metal levels used in the experiment are too low, then the experiment may take an inordinate amount of time to run.

In designing an experiment such as this it is important to state clearly what the objectives are. This is necessary both to save time and to provide the information that can prove or disprove the particular hypothesis being tested, in this case that Cd and Cu, especially in combination, affect tree growth. Thus some form of experimental design is required to obtain the necessary information as to the effects of Cd and Cu would have upon the growth of the trees.

6.1 Choice of Design.

Response surface techniques have been used to determine the effect of ozone and sulphur dioxide upon plants [Ormrod (1984)] and the effect that various heavy metals have upon the growth of plants [Hader et al (1957), Allus et al (1988), Allus et al (1989)]. Response surface techniques have also been used to determine the significance of environmental effects upon the flowering of plants [Armitage et al (1981)].

If only one factor is examined in a particular experimental design, the response may be plotted against the level of the factor. If the factor is examined at two levels then it may be modelled as a straight line. If the design allows three or more levels of the factor to be used, the curvature of the response may also be estimated. For two factors, X_1 and X_2, examined in a 2^2 factorial design, only the main effects and interactions (where the effects of the two factors are not additive) of these factors may be estimated. This is equivalent to measuring the linear slopes in the directions of increasing levels of both

factors and assumes that the response can be approximated by a plane in three dimensions. In a 2^2 design any response in any treatment combination is modelled as

$$\mathbf{Y = B_0 + B_1X_1 + B_2X_2 + B_{12}X_1X_2 + Error}$$

where B_0 is the intercept, B_1 is the slope in direction X_1, B_2 is the slope in the direction of X_2 and B_{12} is the interaction term [Morgan et al (1989)].

Each B parameter is a least-squares estimator. When curvature in the response is small and an appropriate second-order design is used, the model changes accordingly. The expected response when two factors are examined is then

$$\mathbf{Y = B_0 + B_1X_1 + B_2X_2 + B_{11}X_1^2 + B_{22}X_2^2 + B_{12}X_1X_2 + Error}$$

where B_{11} and B_{12} are the parameters of curvature for X_1 and X_2. After all the estimates of these parameters have been made, the responses can be plotted as functions of the explanatory variables by means of a response surface diagram. An experimental design constructed to estimate the coefficient for a model has to meet certain design criteria such as to provide estimates for all parameters, to provide a test for lack of fit, to enable the experiment to be run in blocks and to allow specified variance criteria to be met for estimated parameters and their responses [Morgan et al (1989)]. One of the best designs that meet these criteria is the 3^k factorial design. This satisfies quite a number of the design criteria, but the number of experimental runs necessary is quite large for small numbers of factors even when fractional replicates are used. This problem was overcome by Box and Wilson (1951) by adding star designs to 2^k factorials to produce the central composite design. They are basically 2^k factorial designs with $N_c = 2^k$ factorial points N_o extra points at the centre of the design and $2k = N_a$ extra star points (axial distance 'a'), one at the extreme of each factor and at the centre of the others. So, composites of complete factorials therefore require a minimum of $2^k + 2k + 1$ runs.

A comparison of the minimum number of experimental runs required for K factors in 3^k factorial and central composite design is given in Table 6.1. This clearly shows that the use of the composite designs can provide valuable savings in the number of experiments runs especially when k is large [Bayne and Rubin (1986)].

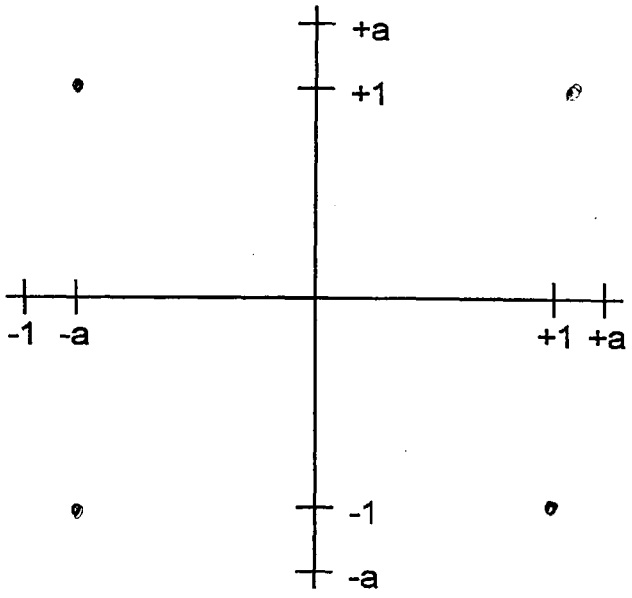
Table 6.1 Numbers of Experimental Runs Necessary for 3^k Factorial and Central Composite Design.

No. of factors k	Treatment combinations Three level factorials 3^k	Composite $2^k + 2k + 1$
2	9	9
3	27	15
4	81	25
5	243	43
5	81 (1/3 factorial)	27 (1/2 fraction)
6	29 (1/3 factorial)	27
6 (1/3 fraction)	243	45 (1/2 fraction)

There are, however some disadvantages, since there are a lower number of degrees of freedom available for estimating the residual error and also some of the effects are estimated using unequal variances.

First of all in constructing a central composite design, certain criteria have to be met. The values of N_C and 'a' have to be determined. The number of centre points should be greater than one in order to estimate the pure error for a lack of fit test. The value of 'a' for the axial points is determined by variance criteria for estimated parameters and responses [Morgan et al (1989)]. With central composite designs it is usual to scale the lower and higher values of the points in the factorial design to -1 and +1 respectively. The centre points then take on the value of zero for each of the factors (Fig. 6.1).

Fig. 6.1. Central Composite Design for Two Factors



The equation below is used to estimate the parameter matrix **B** :

$$\mathbf{Y} = \mathbf{X}\,\mathbf{B} + \mathbf{R}$$

where **Y** is the column of responses, **X** is the matrix of parameter coefficients (which contains all the parameter coefficients for the model), **R** is a column matrix and contains all the unknown residuals, and **B** is the matrix containing the parameters (B_0, B_1) which are to be estimated. To solve the equation, the method of least squares is employed which minimises the sum of squares of the residuals and provides an estimate of the parameters.

The generalised matrix least squares solution for a set of **B** parameters is then given by

$$\mathbf{B} = (\mathbf{X}'\mathbf{X})^{-1}\mathbf{X}'\mathbf{Y}$$

where $\mathbf{X}'$ is the transpose of matrix **X**. The transpose $\mathbf{X}'$ then multiplies **X** to $\mathbf{X}'\mathbf{X}$. The inverse of the matrix is calculated to give $(\mathbf{X}'\mathbf{X})^{-1}$. The inverse pre multiplies $\mathbf{X}'\mathbf{Y}$ to give the solution to the estimated parameters.

If a response surface experiment is repeated under exactly the same conditions, it is unlikely that the same responses would be obtained. Therefore the estimates for b_0, b_1 etc. will be different. They are, however, likely to vary within certain limits that can be estimated. This may be of interest when we are trying to decide whether a parameter is significantly different from zero at a certain level of probability.

To calculate the confidence intervals it is necessary to check initially that all the residuals are normally distributed and that there are no apparent trends. The parameter estimates obtained will usually then vary in normal manner. The variance (s^2) for each parameter can be calculated by multiplying each element of the matrix $(\mathbf{X}'\mathbf{X})^{-1}$ by the estimate of pure experimental uncertainty to form a matrix **V**, the variance- covariance matrix. Along the principal diagonal of **V** (upper left to bottom right) are the variances of the parameter estimates. Each off diagonal value is the covariance between two parameter estimates. Clearly the X values can be chosen in such a way as to minimise the confidence intervals about the b values.

The variance-covariance matrix for a central composite design is determined by the values for N_c, N_a, N_o and the axial spacing 'a'. With the variance-covariance matrix, the variances of the parameters occur down the main diagonal and the covariance's between the parameters as off diagonal elements [Morgan (1991)]. For a two-factor second-order design the matrix for the parameter coefficients appears as:

$$\mathbf{X} = \begin{array}{cccccc} x_0 & x_1 & x_2 & x_1^2 & x_2^2 & x_1x_2 \\ 1 & +1 & +1 & +1 & +1 & +1 \\ 1 & +1 & -1 & +1 & +1 & -1 \\ 1 & -1 & +1 & +1 & +1 & -1 \\ 1 & -1 & -1 & +1 & +1 & +1 \\ 1 & +a & 0 & a^2 & 0 & 0 \\ 1 & -a & 0 & a^2 & 0 & 0 \\ 1 & 0 & +a & 0 & a^2 & 0 \\ 1 & 0 & -a & 0 & a^2 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 \end{array}$$

The $\mathbf{X'X}$ matrix for a central composite design is formed in terms of :-:

$N = N_C + N_a + N_o$ and the axial spacing 'a' as $\mathbf{X'X}$

$$\begin{array}{cccccc} N & 0 & 0 & N_c+2a^2 & N_c+2a^2 & 0 \\ 0 & N_c+2a^2 & 0 & 0 & 0 & 0 \\ 0 & 0 & N_c+2a^2 & 0 & 0 & 0 \\ N_c+2a^2 & 0 & 0 & N_c+2a^2 & 0 & 0 \\ N_c+2a^2 & 0 & 0 & 0 & N_c+2a^2 & 0 \\ 0 & 0 & 0 & 0 & 0 & N_c+2a^2 \end{array}$$

The inverse of this matrix is difficult to calculate by hand and algorithms using computers are usually employed. For the above matrix the inverse is $\mathbf{X'X}^{-1}$

B_0	B_1	B_2	B_{11}	B_{22}	B_{12}
0.196	0	0	-0.107	-0.107	0
0	0.138	0	0	0	0
0	0	0.138	0	0	0
-0.107	0	0	0.194	0	0
-0.107	0	0	0	0.198	0
0	0	0	0	0	0.25

The $\mathbf{X'X^{-1}}$ matrix shows that the covariances between the estimated intercept and the first-order coefficients [COV (b_0b_1) and COV (b_0b_2)] and the second-order mixed coefficients will be zero whereas the covariance between the estimated intercept and the pure second-order parameters, COV (b_0, b_{ii}) will not be zero. Covariances between estimates of pure second-order parameters, COV (b_{11}, b_{22}) will be zero [Bayne and Rubin (1986), Morgan (1991)] if the following criteria is true

$$NN_c = (N_c + 2a^2)^2$$

This condition for uncorrelated, estimated, pure second-order coefficients is true for any number of factors. A central composite design satisfying this condition is an orthogonal design [Morgan (1991), Bayne and Rubin (1986)]. The axial spacing for orthogonality is given by

$$a^2 = \frac{\sqrt{(N_c + N_a + N_o)N_c} - N_c}{2}$$

An orthogonal design (orthogonality ensures that all the effects can be estimated directly and independently of one another) for two factors with $N_c = 4$, $N_a = 4$ and $N_o = 1$ has an axial spacing of a = 1. This is equivalent to a 3^2 factorial design. If the numbers of centre points are increased from 1 to 5 then the axial spacing is increased to 1.267 [Morgan (1991)]. Orthogonality results in the elimination of covariances between the individual estimated pure second-order coefficients. It is possible to use criteria based on the joint effects of all the parameters. Such a criterion is based on variances of estimated

responses for points that are an equal distance from the centre. Therefore it was decided to adopt this type of design.

6.2 Experimental.

The investigation was concerned with the effects that copper and cadmium can have upon the growth, chlorophyll levels, carotenoid levels, needle length and the concentrations of various trace metals in the roots and new growth of young Sitka spruce trees. Since copper and cadmium were the factors to be investigated, a two factor central composite design was used with five centre points. The + 1 and - 1 levels together with the centre point levels of the two factors are given in Table 6.2.

Table 6.2. Concentrations of Cd and Cu Used in the Experiment (mg dm^{-3}).

	Units	-1	0	+1
Copper	Mg dm^{-3}	15	30	45
Cadmium	Mg dm^{-3}	5	20	35

The composite design had N_c = 4 cube points, N_a = 4 axial points and N_o = 5 centre points at an axial spacing of +/- 1.267. The design matrix is shown in Table 6.3

Table 6.3. Uncoded (A) and Coded (B) Design Matrices .

A

x_0	X_1	X_2	X_1^2	X_2^2	X_1X_2
1	45	35	2025	1225	1575
2	45	5	2025	25	225
3	15	35	225	1225	525
4	15	5	225	25	75
5	30	39.005	900	1521.39	1170.15
6	30	0.995	900	0.99	29.85
7	49.005	20	2401.49	400	980.10
8	10.995	20	120.86	400	219.90
9	30	20	900	400	600
10	30	20	900	400	600
11	30	20	900	400	600
12	30	20	900	400	600
13	30	20	900	400	600

Table 6.3 (Contd.)

B

x_0	x_1	x_2	x_1^2	x_2^2	x_1x_2
1	+1	+1	+1	+1	+1
2	+1	-1	+1	+1	-1
3	-1	+1	+1	+1	-1
4	-1	-1	+1	+1	+1
5	0	+1.267	0	+1.605	0
6	0	-1.267	0	+1.605	0
7	+1.267	0	+1.605	0	0
8	-1.267	0	+1.605	0	0
9	0	0	0	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
13	0	0	0	0	0

Thirteen Sitka spruce tree seedlings (2 year old) were potted into a peat substitute (COIR that is composed of coconut husk). The pots that were 12 cm high with a 13 cm circumference had holes in the base to allow for drainage. The trees were allowed to acclimatise to the conditions of growth (16 hour day with an average maximum temperature of 24.5 ^{o}C and an average minimum temperature of 10.5 ^{o}C min.) until new growth appeared on all the trees. Illumination was provided by two four feet warm balanced fluorescent tubes mounted approximately 30 cm above the trees. The trees were fed with a nutrient solution (100 cm^3) every other day. The formulation of the nutrient solution used (Ingestaad (1959)) is shown in Table 6.4. The nutrient compounds were made up into stock solutions and then diluted to give the necessary amount of the individual element. The positions of the trees were randomised every feed day. The trees were kept moist by watering if required on non-feed days. The copper and the cadmium were added as the chlorides. The nutrient solution was selected since it has been used by the Forestry Commission as a standard nutrient formulation for growing Sitka spruce seedlings [Morgan (1983)].

An initial experiment was carried out using acid washed silver sand. This experiment failed since the roots of the trees quickly died. This may be due to a lack of oxygen around the roots, since the sand is quite fine and does not aerate readily.

Table 6.4. Nutrient Formulation, Ingetstaad (1959).

Compound	Conc. (g dm^{-3}) Stock Solution	Element Required	Dilution (100 times) mg dm^{-3}
NH_4NO_3	14.3	N	50
$KH_2PO_4.2H_2O$	4.4	P	10
KCl	2.13	K	50
$CaCl_2.6H_2O$	21.9	Ca	40
$MgSO_4.7H_2O$	15.4	Mg	15
$FeCl_3.6H_2O$	0.5	S	20
$MnCl_2.4H_2O$	0.06	Fe	0.93
H_3BO_3	0.10	Mn	0.17
$CuCl_2.2H_2O$	0.005	B	0.17
$ZnCl_2.2H_2O$	0.004	Zn	0.02
$NaMoO_4.2H_2O$	0.0007	Cu	.002
		Mo	0.003

The experiment was carried out for ten weeks. After this time there was a marked decrease in growth, with many of the trees having long spindly younger needles, with chlorosis observable in the older needles. The length of the apical shoot was measured every 7 days. At the end of the experiment, the following measurements were taken: percentage increase in the length of the apical shoot, the average length of 10 needles, chlorophyll and carotenoid levels in the new growth (determined using the equations developed by Lichtenthaler and Wellburn (1983)], and various trace metal concentrations measured in the needles and roots. The metals were analysed according to standard conditions using atomic absorption spectrophotometry and flame photometry. Needles and roots were dried overnight at 105 ^{o}C (+/- 5^{o}C) prior to trace metal analysis and then digested as previously (Chapter 2). Atomic absorption analysis was performed using a Phillips PU9150 and suitable calibration graphs.

6.3 Results and Discussion.

The results of the experiment are given in Tables 6.5 to 6.12.

Table 6.5. Apical Shoot growth (cm week^{-1})

Tree	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10
1	4.5	6.3	7.4	7.5	8.8	8.0	8.2	8.2	8.2	8.2
2	2.5	4.5	5.5	6.4	6.4	6.4	6.4	6.4	6.4	6.4
3	6.9	10.9	11.4	12.0	13.0	13.4	13.4	13.4	13.4	13.4
4	4.8	7.9	8.5	8.7	9.5	9.6	9.6	9.7	9.7	9.7
5	3.0	6.8	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.3
6	4.9	6.8	7.4	7.8	8.0	8.5	8.7	8.7	8.7	8.7
7	4.5	5.9	6.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
8	1.9	3.0	3.5	4.0	4.8	5.0	5.5	5.5	6.4	6.4
9	2.5	4.4	5.0	5.4	5.8	6.0	6.0	6.0	6.0	6.0
10	2.4	5.7	6.8	8.0	8.5	8.8	9.2	9.2	9.2	9.2
11	3.0	5.9	6.4	8.0	8.0	8.0	8.3	8.3	8.3	8.3
12	1.8	5.0	6.0	7.1	7.7	7.7	7.7	7.7	7.7	7.7
13	2.3	2.6	2.7	4.3	4.4	4.8	6.3	7.7	7.7	7.7

Table 6.6 Total Increase in the Apical Shoot Length and the % Increase in the Apical Shoot and the Average Length for 10 Needles.

Tree	Total Increase (cm)	% Increase	Av. Length of 10 needles (cm)
1	8.2	45.1	1.36
2	6.4	60.9	1.44
3	13.4	48.5	2.05
4	9.7	50.5	1.66
5	7.4	59.5	1.64
6	8.8	44.3	1.86
7	7.0	35.7	1.47
8	6.4	70.0	1.62
9	10.9	69.7	1.55
10	6.0	58.0	1.16
11	9.2	75.5	1.29
12	8.3	63.9	1.29
13	7.7	76.6	1.14

Table 6.7 Chlorophyll and Carotenoid Levels New Growth (Shoots) mg dm^{-3}

Tree	Chl a	Chl b	Carotenoid	a/b
1	1914	697	295	2.75
2	1929	712	281	2.70
3	2364	727	398	3.25
4	2337	859	404	2.70
5	2351	847	407	2.78
6	2069	717	348	2.88
7	2487	883	400	2.82
8	2085	753	355	2.76
9	1995	742	322	2.69
10	1994	723	320	2.76
11	2234	842	347	2.65
12	2054	693	352	2.96
13	1707	705	233	2.40

Table 6.8. Chlorophyll and Carotenoid Levels Old Growth (from stem) mg dm^{-3}.

Tree	Chl a	Chl b	Carotenoid	a/b
1	1385	527	281	2.62
2	1423	626	272	2.27
3	1409	564	433	2.50
4	1503	525	268	2.86
5	1601	533	316	3.00
6	1302	724	454	1.80
7	1560	846	515	1.84
8	1324	742	346	1.78
9	2029	683	338	2.97
10	1751	588	338	2.98
11	1910	623	368	3.07
12	1688	536	328	3.14
13	1326	476	354	2.78

Table 6.9 Metal Levels in the New Growth (mg dm^{-3}).

Tree	Ca	Cu	Cd	Mg	Zn	Fe	Mn	K
1	2211	7.5	0.07	98.4	155.8	28.6	81.5	2207
2	1501	6.3	0.25	26.4	460.6	24.4	32.2	881
3	1969	10.1	1.29	72.9	375.3	24.6	48.4	1754
4	2560	7.5	0.17	124.1	559.9	39.3	87.1	2189
5	2695	10.1	0.08	111.4	515.1	81.3	75.9	2582
6	2169	7.3	0.23	229.5	66.8	30.6	83.6	206
7	2006	6.2	0.21	105.9	91.6	36.5	67.2	198
8	2365	7.9	0.26	124.8	297.8	46.7	183.9	2019
9	2520	12.2	0.68	102.7	565.8	23.1	55.4	2177
10	2855	12.0	0.23	120.3	518.2	26.4	59.4	2766
11	2238	7.4	0.77	97.2	595.7	17.7	70.9	2087
12	2105	10.0	0.19	76.8	583.9	30.3	45.1	2082
13	2565	9.4	0.24	93.5	531.8	18.8	68.4	2225

Table 6.10. Metal Levels in the Roots (mg dm^{-3})

Tree	Ca	Cd	Cu	Mg	Zn	Fe	Mn	Na	K
1	1028	52.07	11.7	153.1	167.3	785.5	25.0	84	2867
2	940	2.26	9.9	164.9	504.5	1277.9	11.4	432	2251
3	534	32.57	8.6	112.4	285.8	894.3	10.4	219	1879
4	808	2.05	11.8	117.6	523.2	1050.6	8.6	346	2213
5	748	33.50	9.9	117.4	31.0	958.9	9.0	332	1753
6	759	0.85	7.8	115.5	425.6	1086.2	9.9	668	1979
7	855	36.38	14.5	181.7	197.7	2171.7	12.7	620	2030
8	725	32.23	12.0	139.0	321.1	1366.5	9.9	427	1786
9	742	11.99	8.1	162.3	444.0	1443.4	11.1	106	1502
10	811	23.81	9.3	105.8	464.1	430.7	9.0	262	2280
11	715	12.53	9.5	101.1	492.4	1018.1	13.2	450	1792
12	710	30.04	7.7	132.9	487.3	1268.9	12.0	295	1677
13	959	34.03	14.1	145.7	535.7	1144.6	9.5	626	2578

The only measurements that were statistically significant were the average needle length (Table 6.11), and cadmium levels in the root (Table 6.12). Analysis of variance (ANOVA) was performed using a program written by Morgan (1991). It provided data for the t-test on each of the individual parameters, and provided information on the lack of fit for the equation. The remaining results are given in Appendix 5. These were statistically insignificant.

Table 6.11. Average Length of Ten Needles (mm)

(a)

Tree	Response (Y)	Estimated (Y)	Residuals
1	13.6	14.037	-0.437
2	14.4	16.300	-1.900
3	20.5	19.438	1.062
4	16.6	17.001	-0.401
5	16.4	17.033	-0.633
6	18.6	16.923	1.677
7	14.7	12.995	1.705
8	16.2	16.861	-0.661
9	15.5	12.942	2.558
10	11.6	12.942	-1.342
11	12.9	12.942	-0.042
12	12.9	12.942	-0.042
13	11.4	12.942	-1.542

(b) Analysis Of Variance

Source	Sums of Squares	df	Mean Squares	F Ratio
Total	0.8516	12		
Regression	0.6279	5	0.126	3.929*
Residual	0.2237	7	0.032	
Lack of fit	0.1165	3	0.039	1.448
Pure error	0.1073	4	0.027	

(c)

Coded Parameters	Estimate	t-Statistic *	Uncoded Parameters	Estimate
b 0	1.294	16.341	b 0	2.122
b 1	-0.153	2.292	b 1	-0.033
b 2	0.004	0.065	b 2	-0.029
b 1 1	0.124	1.571	b 1 1	0.001
b 2 2	0.251	3.193	b 2 2	0.001
b 1 2	-0.118	1.315	b 1 2	0.001

Coded Stationary Point	Uncoded Stationary Points
0.689 x 1S	40.337 x 1S
0.152 x 2S	22.286 x 2S
Response at Stationary Point	**Response at Stationary point**
= 12.42	= 12.42

* 90% significance

From the analysis of variance table, for the average needle length the sum of squares due to the regression as a percentage of the total sums of squares is 73.7%, showing that quite a large proportion of the variation in needle length is accounted for by the regression equation. The regression equation has to account for a large and significant part of the variation, otherwise it will not give a reasonable representation of the data to which it is fitted. One test of this ratio is the correlation coefficient r. A high value of r can give an impression of a good fit to the model that is not necessarily true. More usually an F-test is applied, since this will take into account any shift in the number of degrees of freedom from the residual sum squares or to the factor or regression sum of squares.

The variance ratio of the regression mean square to the residual mean square ratio gave 3.929. The residual variation in response surface experiments can be broken down into two components which are the lack of fit and pure error. The sum of squares due to pure error is calculated from the variation in response of the five centre points. The sum of squares due to lack of fit is calculated by subtracting the pure error sum of squares from the residual sum of squares. The variance ratio of these mean squares is 1.448, which shows that the model is a fair approximation of the data. The residual mean square (0.032) is used to give an estimate of the error variance in the calculation, useful for deciding the importance of individual parameters in the t-statistic.

The stationary point may not necessarily be a minimum or a maximum. It is also possible for the stationary point to lie outside the experimental region defined by the factor levels. However, the approximating response function is only expected to be adequately described in the immediate neighbourhood of the experimental design. This is true for this response since it represents a minimum and this is clear from the uncoded plot (Fig 6.2). Therefore, a combination of the two metals at this point is bringing about the lowest needle length.

The uncoded plot shows that there is little variation in needle length with variation in the copper levels, but at high levels of cadmium and low levels of copper there is an increase in the needle length. Cadmium is known to be a toxic metal, and the experiment did show chlorosis in the older needles (a symptom of Cd toxicity) and an apparent stopping of growth. In spite of what the statistic for the model suggests, this result is difficult to interpret since Cd is not an essential element. A possibility may exist in that the Cd may be affecting various enzyme systems within the foliar tissue, and this may cause the resulting spindly growth of the needles.

Fig 6.2. Response Plot for Needle Growth (mm).

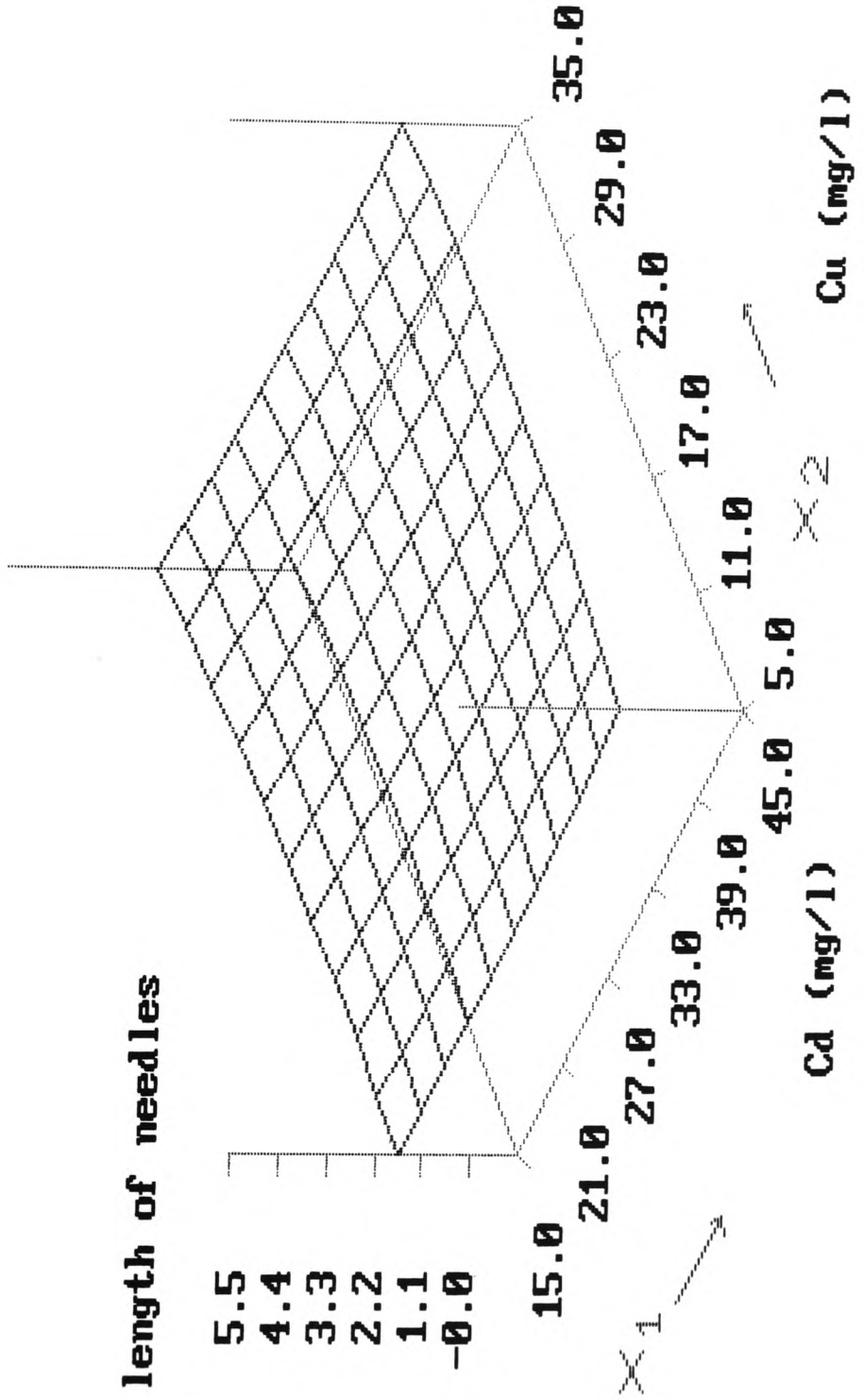


Table 6.12 Results for the Cadmium Levels Found in the Roots (mg dm^{-3}).

(a)

Tree	Response (Y)	Estimated (Y)	Residuals
1	5.21	4.9055	0.3015
2	0.23	0.5655	-0.3395
3	3.26	3.2485	0.0085
4	0.21	0.8375	-0.6325
5	3.35	3.6497	-0.2997
6	0.09	-0.6271	0.7121
7	3.64	3.6630	-0.0250
8	3.22	2.7855	0.4375
9	1.20	2.2805	-1.0815
10	2.38	2.2805	0.1005
11	1.25	2.2805	-1.0280
12	3.00	2.2805	0.7235
13	3.40	2.2805	1.1225

(b) Analysis Of Variance

Source	Sums of Squares	df	Mean Squares	F Ratio
Total	30.7145	12		
Regression	25.2995	5	5.060	6.541*
Residual	5.4151	7	0.774	
Lack of fit	1.3951	3	0.465	0.463
Pure error	4.020	4	1.005	

(c)

Coded Parameters	Estimate	t-Statistic *	Uncoded Parameters	Estimate
b 0	2.280	5.852	b 0	0.013
b 1	0.346	1.057	b 1	-9.602
b 2	1.688	5.153	b 2	1.223
b 1 1	0.588	1.518	b 1 1	-0.336
b 2 2	-0.479	1.237	b 2 2	-0.034
b 1 2	0.482	1.097	b 1 2	0.023

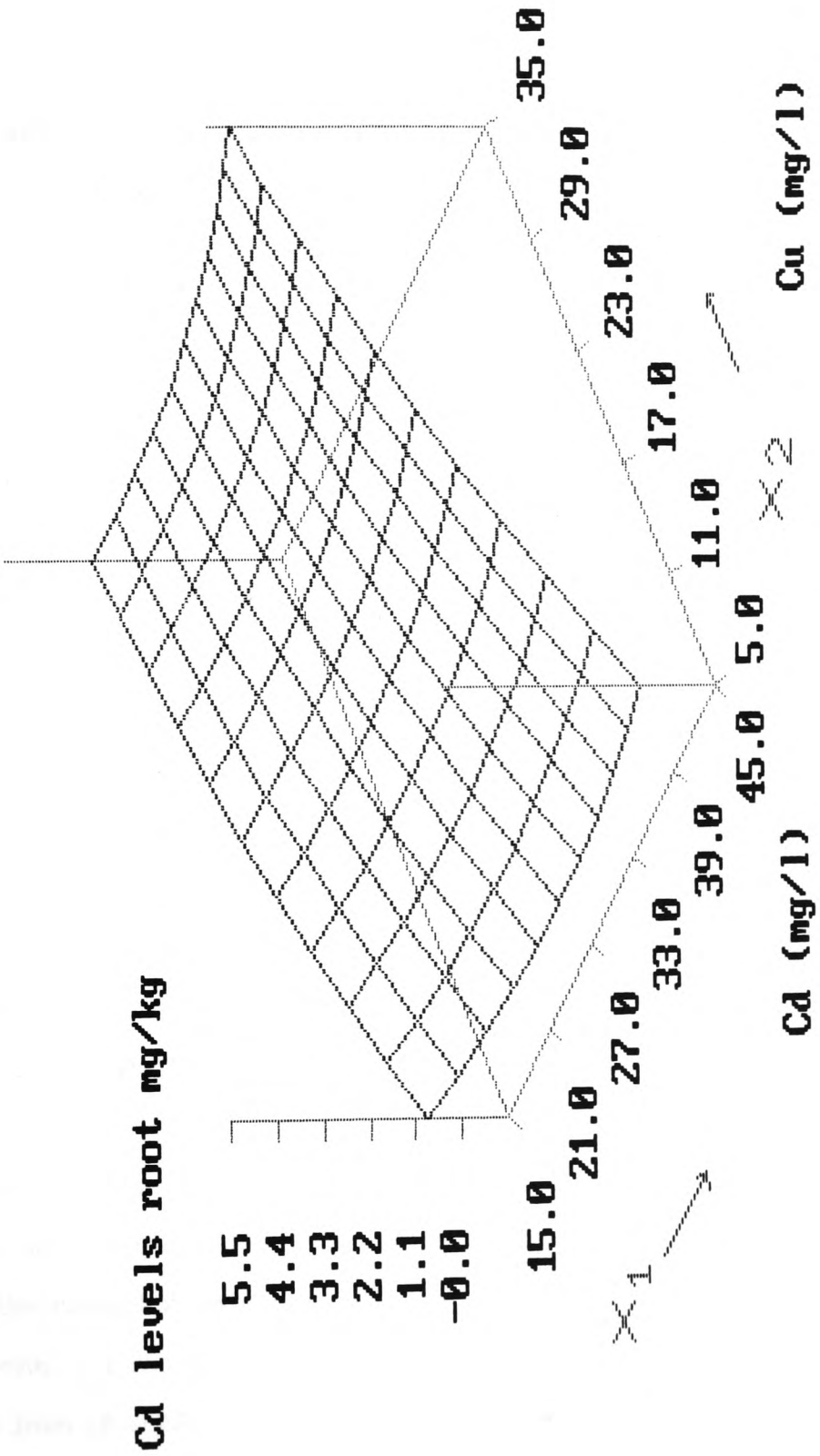
Coded Stationary Point	Uncoded Stationary Points
-0.843 x 1S	17.370 x 1S
1.337 x 2S	40.050 x 2S
Response at Stationary Point	**Response at Stationary point**
= 3.263	= 3.263

*95% Significance

The analysis of variance table, the sums of squares due to the regression as a percentage of the total sums of squares being 82.4% showing that a reasonable amount of the variance is being accounted for by the regression equation for the Cd levels in the roots. The variance ratio of the regression mean square to the residual mean square ratio gave a value of 6.541, which is significant at the 95% level. The lack of fit ratio is 0.463 showing the model to be a good approximation of the data.

The stationary point co-ordinates do not lie within the boundaries of the plot. The results here (Fig 6.2) indicate that the Cu is not having any effect on Cd uptake, even at high levels of Cu. This may be due to the levels of Cu and Cd chosen for the design may be at such a toxic level as to have damaged the various metal uptake mechanisms in the roots. It has been suggested that copper at high levels can inhibit the uptake of cadmium [Burton et al (1986)]. Workers have shown that plant Cd levels can be reduced by additions of Cu [Cataldo and Wilding (1978)] and that the Cd levels in lettuce leaves were reduced by additions of copper to the soil [Lepp (1981)].

Fig 6.3. Response Plot for the Cadmium Levels in the Roots.



CHAPTER 7

7. Discussion and Conclusions.

The Forestry Commission selected twenty sites at the Afan 1 Forest that covers a wide range of growth rates and soil types. Visually, there are obvious signs of decline at many of the poor sites of the Afan 1 forest. Bent top is evident in many of the trees at the poorer sites.It is possibly an effect due to a decrease in the vigour of the tree. The poor sites canopy is thin. This is due in part to the loss of needles and also due to the production of few needles, of small needles and because few shoots are produced to bear needles. Needle loss is generally preceeded by various types of discolouration. This needle discolouration is variable in the poorer stands. Some of the discolouration or chlorosis is characteristic of potassium deficiency. Some discolouration is due to attack by *Elatobium.* Needles attacked by *Elatobium* sometimes turn bronze and then fall. With trees in decline, shoot die-back is most noticeable in the lower crown. It is possibly due to the loss of foliage and the inability of the weaker shoots to flush in spring. In severe cases of decline at the Afan 1 Forest, individual trees die completely. The average annual rainfall for the forest is 2400 mm. 1984 was considered to be dry with only 2310 mm rain and 1986 was considered to be wet, with 3182 mm. Some of the sites have drainage problems with the problems of water logging that ensue.

The soil types of the Afan 1 Forest are: Surface water gley (1 site), Peaty gley (9 sites), Ironpan soil or peaty ironpan soil (6 site) and deep peat (4 sites). The peaty and non-peaty gleys have a fluctuating but permanent water table. Some of the deep peat sites exhibit very poor growth. The rooting depth on the peats is shallow (10-25 cm). On the peaty gleys it ranges from 18 to 40 cm and on the ironpans from about 20 to > 60 cm; shallow

rooting can lead to trees toppling in high winds and when they get too tall [Coutts (Pers. Comm.) (1989)]. A full list of the soil types for each site is to be found in Appendix 7.

One of the visible symptoms of forest decline is the production of epicormic shoots from branches and the main stems [Rose (1985)]. It is assumed that these epicormics are produced in response to defoliation. Experimental defoliation, carried out by the Forestry Commission, has produced epicormic shoots in trees, apparently as a result of the release of dormant buds from apical dominance [Coutts Pers Comm. (1989)]. At the Afan 1 forest, these shoots appear greener than their non-epicormic counterparts.

The survey performed in 1987 was to determine whether there were any significant differences in the chlorophyll content between epicormic shoots and non-epicormic shoots. There were no significant differences found between the two types of growth for chlorophyll content or the metals that were analysed. The apparent 'greener' appearance of the epicormics may be attributed to them being made 'more visible' on trees that have a greater degree of defoliation of the normal growth.

From Chapter 2 (1986 survey) it is seen that there are two clearly defined tree growth types, namely the good growth sites and the poor growth sites. This was revealed using cluster, PCA and SIMCA analyses on the results obtained on the foliar levels of chlorophyll and various metals for one, two and three years old Sitka spruce needles. A not too dissimilar result was found for the 1987 survey (Chapter 3) when PCA and SIMCA were used only on the one year old needles from epicormic and non-epicormic growth. Pattern recognition methods have been successfully employed in the evaluation of forest vitality [Simmeilt and Schulten (1989)]; here spruce trees were successfully classified as to their health on the basis of data provided by pyrolysis-field ionisation mass spectrometry.

In Chapter 2, a Student's t-test revealed the Good growth sites to have higher amounts of potassium in the year 1, 2 and 3 needles. Also, the good growth sites had significantly higher levels of calcium in the year 1 and year 2 needles. Table 7.1 shows the decline of potassium levels over the three needle ages for the good and poor growth sites. The good growth sites show a limited potassium loss using the year 1 needles as 100%) as compared to the poor growth sites. A not too dissimilar result was found for the 1987 survey (Chapter 3) when PCA and SIMCA were used only on the one year old needles from epicormic and non-epicormic growth.

No statistically significant differences were found for the chlorophyll levels between the good and poor growth sites. The highest chlorophyll a and b levels were found in the year 2 growth, with both the years 2 and year 3 needles having higher levels than the youngest needles. The work carried out on the pigment levels of Norway spruce by Blintsov and Asyutin (1983) also found the year 1 needles to have lower levels of pigments than the year 2 and year 3 needles.

Table 7.1. Comparison of Potassium Levels for the Needles of the Three Growth Years for the Good and Poor Growth Sites at the Afan 1 Forest.

	Good Growth		Poor Growth		
	K (mg/kg)	% diff.	K (mg/kg)	% diff.	Sig. diff. Good/Poor
Year 1	5752	0.0	4960	0.0	**
Year 2	5328	-7.4	3613	-27.1	****
Year 3	4401	-23.1	2866	-42.2	****

** 95% Significance
**** 99.9% Significance

The good growth sites show a greater loss of magnesium over the three needle ages. This is shown in Table 7.2.

Table 7.2. Comparison of Magnesium Levels for the Needles of the Three Growth Years for the Good and Poor Growth Sites at the Afan 1 Forest.

	Good Growth		Poor Growth		
	Mg (mg/kg)	**% diff.**	**Mg (mg/kg)**	**% diff.**	**Sig. diff. Good/Poor**
Year 1	1034	0.0	1083	0.0	n.s.
Year 2	724	-20.0	759	-30.0	n.s.
Year 3	501	-51.5	815	-24.7	***

*** 99.0% Significance

Calcium also gave a decrease for the good growth sites over the three growth years, again using the year 1 needles as 100% (Table 7.3).The poor growth sites showed an increase in the calcium levels over the three growth years.

Table 7.3. Comparison of Calcium Levels for the Needles of the Three Growth Years for the Good and Poor Growth Sites at the Afan 1 Forest.

	Good Growth		Poor Growth		
	Ca (mg/kg)	**% diff.**	**Ca (mg/kg)**	**% diff.**	**Sig. diff. Good/Poor**
Year 1	3229	0.0	1923	0.0	**
Year 2	3946	+22.2	2227	+15.8	**
Year 3	2870	-11.1	2922	+51.9	n.s.

** 95% significance

The nutrient supply, that is the supply of minerals such as calcium, magnesium, potassium and in some cases zinc has been suggested to play a major role in the forest injury problem. Deficiencies of these elements have been observed in many injured forest stands [Zottl et al (1977), Prinz et al (1982)]. Nutrient deficiency has been shown to affect shoot and needle elongation in Sitka spruce [Chandler and Dale (1990)]. In four years old needles from spruce trees, Zottl and Mies found the magnesium content to be less than 200 ppm [Guderian et al (1985)]. This was significantly less than the amount found in needles from visibly healthy trees. A similar relation was found by Zech and Popp (1983), where they found the younger needles in the upper crown of diseased spruce trees

contained 250-270 ppm of magnesium compared to the 530-590 ppm in the control samples. Visible symptoms of magnesium deficiency in spruce occur when the needle tissue contains less than approximately 350 ppm [Guderian et al (1985)].

The good and poor growth sites apparently do not appear to be deficient in magnesium (see Table 7.2.). In both cases, there is a decrease in the magnesium content over the three growth years examined. It has been established that magnesium and calcium deficiencies occur on acid soils, which has lead to the assumption that the inputs of atmospheric acidic compounds promote the leaching of alkaline (Group I metals) and alkaline-earth (Group II metals) cations [Guderian et al (1985)].

The good growth sites appear to have lost calcium whilst the poor growth sites have not (Table 7.3). The differences in the calcium needle content were found to be significantly higher in the year 1 and year 2 needles for the good growth sites, even though the good growth sites appear to have the loss whilst the poor growth sites have gained calcium over the three growth years.

The poor growth sites have lost the greater percentage of potassium over the three growth years. Artificial rain studies have shown that decreasing the pH increased the foliar leaching of potassium from sugar maple and bean leaves prior to the appearance of visible injury [Wood and Bormann (1975)]. Acid fog appears to be more common at higher elevations. It is believed that acid fog can be more harmful to vegetation, by causing the leaching of nutrients from foliage even if it has the same pH as rain [Prinz et al (1982)]. In the Black Forest in Germany, there appears to be a slight correlation between forest decline and hydrogen ion deposition [Prinz et al (1987)].

There have been many reports of increased leaching of nutrients from foliage associated with acid precipitation [Wood and Bormann (1975), Tamm and Cowling (1977) Richter et al (1983), Scherbatskoy and Klein (1983]. Increased leaching has been observed at pH levels where no visible foliar damage has been observed. It has been suggested that ion exchange was occurring [Richter et al (1983)]. The most dramatic increases in leaching have been observed with damaged tissues. This takes place when the pH is less than 3.3 [Berg (1987)].

Exposure of needle or leaf cells to acid precipitation is minimised by the presence of the plant cuticle. This is a waxy layer that covers the needles and leaves. Direct acid damage to foliage does occur but seldom above pH 4.0 due to the presence of the intact cuticle [Berg (1987)]. The cuticle does allow movement of protons into the leaf and mineral ions out of the leaf but only at very low rates. Most of the damage done to the cuticle appears to be a secondary effect associated with the loss of support from injured cells in the underlying tissues. This could enhance the leaching of nutrients from leaves or needles by acid precipitation when the tissues that underlie the cuticle are damaged [Berg (1987)].

Other workers have shown that when the cuticle is damaged the plant may lose frost and drought resistance [Percey and Baker (1990)]. The waxy cuticle may also be damaged by airborne pollutants such as ozone [Percey et al (1990)]. Ozone has been implicated in causing damage to tissues within the needles or leaves of plants [Schmitt and Ruetze (1990)].

In experiments using open topped chambers Schmitt and Ruetze showed ozone and sulphur dioxide to cause changes in the vascular bundles of spruce and fir trees in combination with acid rain. The sieve cells of phloem showed increased damage. The work also revealed that the walls of young adaxial sieve cells lacked the typical thickening

usually observed in naturally aged needles. This may cause problems for the translocation and assimilation of nutrients within the plant [Schmitt and Ruetze (1990)]. Workers have not been able to reproduce the visible damage symptoms of decline in trees used in chamber experiments. This is sometimes due to the design of the chambers used. Also it is difficult to set up the experiment to reproduce the effects of photochemical pollution at altitude on plants, since it is not easy to reproduce the type of electromagnetic radiation to be found at the higher elevations [Arndt et al (1990)].

The eight month survey of the pair of trees at site 15 (Chapter 4) also adds credence to the possibility of nutrients being leached out of the foliage. The two trees both showed a general decline of most elements that were analysed over the eight month period (Table 7.4).

This survey also revealed a pattern over the eight months in the levels of chlorophyll 'a' and chlorophyll 'b'. For both trees there is a clear maximum in November for chlorophyll 'a' and chlorophyll 'b'. Some workers examining the pigment levels in Norway spruce have found that the chlorophyll levels peak in spring and winter [Stagfelt (1927), Zacharowa (1929)]. Atanasiu (1968) followed the trends of chlorophyll concentrations in various conifers from October to April and found that the highest concentrations were obtained at the end of November.

Table 7.4. Percentage Change in Foliar Element Levels for the Pair of Trees at Site 15.

	Good Growth	Poor Growth
Element	% Change	% Change
K	-37.8	-54.4
Mg	+4.1	-12.7
Ca	-23.8	-41.3
Cu	-21.1	81.5
Ni	-46.7	38.3
Zn	+57.9	-80.7

The good growth tree appears to have lost less of its minerals over the eight month time period as compared to the poor growth tree. Site 15 is one of the higher elevation sites (515m) so it may be experiencing conditions similar to those in other parts of Europe where decline is more prevalent at higher elevations [Treshow and Anderson (1989)].

The decrease in the foliar element levels as suggested by the results in chapters 2 and 4 may be due in part to acid fog, the so called 'occult' mist. Occult deposition occurs mainly at higher elevations, where natural stresses are also more severe [Unsworth and Crossley (1987)]; so the effects of the acid mists may be more of a problem at the higher elevations at the Afan 1 Forest. In fact the sites at the higher elevations exhibited the poor growth. Field experiments suggest that exposure to atmospheric sulphur increases frost sensitivity in grasses [Davidson and Bailey (1982)].

Increased atmospheric acidity has also been shown to reduce the freezing resistance of spruce [Treshow and Anderson (1989)]. Occult deposition can result in large inputs of protons, sulphate and nitrate into vegetation and soils; evaporation and concentration on leaf or needle surfaces may concentrate the pollutants and lower the pH of the droplets [Unsworth and Crossley (1987), Leith et al (1989)]. The pH of acid fog has been shown to be lower than that of rain waters. One study showed values to be as low as 2.5 [Scmitt (1986)].

Combinations of acid mist and ozone in fumigation experiments on young Norway spruce caused a decrease in the chlorophyll content of the older needles in some of the trees used [Senser et al (1990)]. The experiment also showed that there were increases in the violaxanthin content upon fumigation with ozone. This increase may be due to the protective function of this pigment [Senser et al (1990)]. Pfirrman et al (1990) conducted experiments on the effects of ozone and acid mist on clones of Norway spruce. The

results they obtained showed that there was not significant loss of minerals from the needles.

Apart from the possible direct effects of acid deposition on forests, the effects upon tree growth can be considered as a nutrition problem. The increased deposition of nitrogen can be regarded as a fertiliser effect [Nihlgard (1985)]. Increased deposition of inorganic nitrogen from the atmosphere will most likely increase tree growth. The long term effect of the deposition of inorganic nitrogen may be comparable to the long term effects of incomplete fertilisers. Initially there is an increase in the rate of growth that then leads to an accumulation of toxic waste products because the balance between non-nitrogen (carbohydrates) and nitrogen containing metabolites (proteins) is disturbed and valuable minerals such as potassium, calcium and phosphate are used up by the plant in an attempt to re-establish a balance [Nihlgard (1985)]. This can lead to deficiencies in elements such as potassium, magnesium and phosphorus and this may be strengthened by increased leaching caused by acid precipitation [Abrahamsen (1980)].

It has been shown though, that ozone can have an effect upon the yield of plants [Wellburn (1988), Keller and Matyssek (1990)]. Ozone can reduce the rate of photosynthesis and this can have a detrimental effect upon the growth of the plant. This is shown in the experiments on oat plants by Myhre et al (1988). Ozone has been shown to be a major contributing factor to forest damage in the United States [Ashmore et al (1985)].

Ozone has been suggested as a possible cause to enhanced leaching of nutrients from foliage as well as acid rain. This hypothesis suggests that the ozone damages the various membranes found within the plant cells and this allows the leaching of nutrients from the needles or leaves. Experiments have shown that ozone can damage the waxy cuticle that

covers leaves and needles [Percey et al (1990)]. This can lead to various problems for plants such as a decreased resistance to drought and frost [Percey and Baker (1990)]. Also, if ozone enters the needles or leaves of a plant, it may come into contact with ethylene or hydrocarbons within the tissues that can result in the formation of free radicals [Wellburn (1988)]. These free radicals can further damage the waxy cuticle and the waxy plugs that are found covering the stomata and this can lead to problems of excess transpiration and gas exchange [Wellburn (1988)].

Studies to confirm or disprove the 'ozone' hypothesis are conflicting. Experiments involving the fumigation of trees with ozone have produced varied results. Prinz et al (1987) quote an experiment where whole trees were intermittently treated with ozone at various levels (0 $\mu g\ m^{-3}$, 200 $\mu g\ m^{-3}$ and 600 $\mu g\ m^{-3}$) and artificial acid fog. The experimental result suggests that ozone enhanced the leaching of magnesium, calcium, potassium zinc, ammonium and sulphate. The results also suggest that the efflux of cations is dependent on ozone concentration, pH, previous damage to needles and needle age. Other workers have found little or no evidence that ozone enhances leaching of nutrients from needles [Skeffington and Roberts (1985), Keller and Matyssek (1990)]. Some tree species such as Norway spruce are tolerant of ozone and are classed as such in the United States [Wellburn (1988)].

Why there are differences of some of the nutrients in the foliar material of the good and poor sites may also be attributable to the poor growth trees being denied the nutrients in the first place. There may be increased leaching of nutrients from the soils at the poor sites that are at the higher elevations. The nutrients may be washed out of the soils more easily at the higher elevations. The soil solution analysis (Chapter 2) does not provide much evidence for this though. There is no significant difference in the pH levels of the poor growth (pH 3.8) and good growth sites (pH 4.1). In most cases the soils at the Afan

1 forest are peat. This means the soils in many cases are acidic in nature due to the high organic material content.

The differences in growth exhibited at the Afan 1 forest is most probably due to a combination of different factors. The droughts of the mid 1970's and those of the 1980's most probably played an important part in the forest decline seen in many parts of Europe. Thus drought combined with various pollutants would have a detrimental effect on trees. The trees weakened by the drought would find it difficult to cope with other stresses [McLaughlin (1985), Rose (1985)].

The various effects of ozone , acid rain and acid mist (fog, occult), nitrogen deposition etc.. will all take their toll. Trees may be able to withstand any one of these stresses singly but a mixture of these stresses may be too great for some trees. This then brings up the question of why some trees (same species) should show signs of decline whilst others are quite healthy; as observed at site 15 with the pair that was studied here. It is quite possible that some of the Sitka at the Afan 1 forests are more tolerant to the growing conditions found at the forest. Evolution of resistance by plants is a well-known phenomenon and it is possible that plants may grow and survive in the presence of high concentrations of 'heavy metals' or salts or periods of prolonged drought [Wellburn (1988)]. Norway spruce is known to be ozone resistant [Wellburn (1988)], while tobacco is a good biological indicator for ozone pollution [Bell (1984)].

Resistance to pollution by plants has been defined by Bradshaw as 'the ability to maintain growth and remain free from injury in a polluted environment'. [Wellburn (1988)]. There may be two types of Sitka spruce at the Afan 1 forest. The one class may possess stress tolerance due to the possession of genes that confers a set of characteristics that give an improved ability to detoxify, repair or compensate for injury caused by atmospheric

pollutants. The other class type may not posses these vital genes. Even though at the Afan 1 forest the poor growth sites are to be found at the higher elevations, there are still trees at these elevations that are surviving remarkably well. This is shown clearly in the pair of trees studied at site 15, where one of the pair is over forty feet high and very healthy, and the other is stunted and going into severe decline. It must also be stressed that some of the poor sites are to be found at elevations comparable with the good growth sites (See Table 2.17). Table 7.5 shows a t-test between the foliar levels of chlorophyll and metals for the 1986 needles and the 1987 one year old needles.

Table 7.5. Student t-test for the 1986 and 1987 One Year Old Needles (critical t-value 2-tail at 95% Probability)

Var.	1986 Mean	1987 Mean	1986 s^2	1987 s^2	Corr.	Pooled Variance	d.f.	t-value	2-tail Prob.	Critical t value
Chl a	1033	710	94218	20193	0.56	24561	18	2.1	0.00	2.10
Chl b	322	208	12209	2486	0.43	2374	18	4.97	0.00	2.10
K	5335	4462	5234347	1600586	0.26	28903	18	2.97	0.01	2.10
Ca	2542	1883	1728979	442173	0.31	267113	18	2.24	0.04	2.10
Mg	1059	1327	46443	59049	0.57	29704	18	-5.45	0.00	2.10
Cu	2.5	4.7	2.3	4.3	-0.03	-0.11	18	-.3.75	0.00	2.10
Cd	6.6	0.7	141.2	1.1	0.07	0.90	18	2.17	0.04	2.10
Ni	6.29	6.02	15.13	8.6	0.05	0.63	18	0.25	0.80	2.10

The results of the t-test for the 1986 and 1987 needles show 1986 to be significantly higher for chlorophyll 'a ' and 'b', potassium, calcium and cadmium. The 1987 results have a significantly higher level of magnesium whilst there is no significant difference for nickel. Why there is such a difference for one year old needles sampled at two different years is hard to say. Not much information can be made from a comparison such as this as the a data base is too small. This information needs to be taken over a longer period say 5-10 years and then trends in the data may be established.

Since some workers suggest that there are genetic differences between trees of the same species that may make them better suited to severe growing conditions [McLaughlin (1985), Wellburn (1988)], it was decided to try and determine if this was the case at the Afan 1 forest. Peroxidase occurs as isoenzymes and this enzyme has been successfully used for the identification of individual plants [Baaziz and Saidi (1988)] and as a means of determining the genetic variability of populations of plants [Proctor et al (1989)]. Another reason for looking at peroxidases is due to the fact that its activity has been shown to be enhanced by high levels of cadmium and fluorine.

There are many pairs of trees growing at various sites in the Afan 1 forest. These pairs of trees were planted at the same time at their respective sites and are supposedly from the same seed stock. The pairs are noticeable as one member of each pair is healthy with strong growth and good foliage whilst the other is stunted with poor foliage. These trees and grafts from them were sampled and examined for peroxidase activity. The enzyme extract was subjected to PAGE to see if there are any similarities or differences in the banding from the trees of each pair and their grafts.

The results of the experiment provided very little useful information. The grafts had different banding patterns to the trees they were taken from. There are many reasons for this. It may be due to fungal or insect attack pollution or just periods of cell wall manufacture [Spanu and Bon-Fante Fasolo (1988)]. Since this was a field experiment then there are many factors that are outside experimental control and as such may have lead to poor experimental result.

Chlorophyll is of vital importance to plants since it is the major component of the photosynthetic apparatus [Stryer (1988)]. Chlorophyll 'a and 'b' occur in a ratio of approximately 3:1 [Lichtenhaler (1977)]. This a/b ratio can be modified by environmental

factors and growth conditions [Lichtenthaler (1983)]. Research has shown that photooxidants can damage chlorophyll that may or may not affect the a/b ratio. Anything that can cause free radicals within the needle or leaf tissue can reduce chlorophyll levels. Ozone can promote free radical formation within plant tissue [Wellburn (1988)]. Also chlorophyll lends itself to be analysed reasonably readily from samples from actual field conditions, providing the samples are kept from the light and preferably under liquid nitrogen [Cape et al (1988)].

Analysis of plant pigments can be determined in via two major methods, namely spectroscopically using various sets of equations based on the molar absorptivities of the pigments (SCOR-UNESCO) or by chromatographic techniques. Of the two methods chromatographic separation lends itself to the more accurate of the two methods, but this is the slower of the two. The spectroscopic methods can be used in the field via the use of portable equipment. So, a calibration of the SCOR-UNESCO equations was attempted to determine their accuracy and to provide any additional information on them. These equations rely on absorption measurements being made on the total extract from the foliar material. Thus there may be material present that may be additive to the absorbances determined for the chlorophylls. In fact the chlorophylls themselves have absorbances that overlap. Another reason for performing this piece of work was to try and provide a cheaper alternative to HPLC.

The results obtained from the experiments proved to be quite promising. The SCOR-UNESCO equations apparently are underestimating the chlorophyll 'a' concentration. The result for chlorophyll 'b' was disappointing since the detector used (UV/Vis) was giving a poor response for chlorophyll 'b' (See Fig. 5.6 Chapter 5). Though the result does suggest the SCOR-UNESCO equations may be reasonably accurate in their determinations of chlorophyll 'b' concentrations.

The separation of the chlorophylls from an extract of pigments taken from Sitka spruce needles was effected using an isocractic system of acetonitrile. The column used was a C-18 reversed phase type. The method employed does use methanol in sample preparation or analysis since methanol is capable of causing allomerization of both chlorophyll 'a' and chlorophyll 'b' and both of these allomers show up in chromatograms [Hynninen (1979a and 1979b)]. The allomers of both chlorophylls are C-10 epimer and they function the same as the parent chlorophylls except that there is a slight change in their absorption maxima and retention times on C-18 reversed phase columns [Hynninen (1979a and 1979b)].

The analysis time (around 17 minutes) compares quite favourably with some of the published work on the separation plant pigments using HPLC where analysis times have been in excess of 20 minutes [Shoaf (1978), Scholz and Ballschmiter (1981)].

Going on from HPLC, a more recent piece of hardware to come into the chromatographer's armoury is SFC (Supercritical Fluid Chromatography). This technique lends itself to faster separation and times better resolution as compared to HPLC. A method for the separation of chlorophyll 'a' and 'b' from each other was developed for SFC see Chapter 5). The method developed used an amine packed column with carbon dioxide as the supercritical liquid and a mixture of 97% methoxymethanol/3% methanal as modifier. The methanal was used to change the conditions of the amine column thus effecting a novel method of separating these two photosynthetic pigments. The method is capable of further development, such as developing a supercritical fluid extraction method linked to on line analysis of the pigments. SFC lends itself to being interfaced with mass spectrometers that can be used to determine low concentrations with good accuracy.

Acid rain can lead to the acidification of soils [Wellburn (1988), Treshow and Anderson (1989)]. This causes the soil pH to fall thus releasing 'heavy metals' into the soil solution. Once mobile these 'heavy metals' are then free to be taken up into plants. The results from the 1986 survey (chapter 2) gave indications at some of the sites of elevated levels of cadmium. Cadmium is well known for its toxic effects on living systems [Page et al (1981)]. Cadmium has been shown to cause root death in plants [Godbold et al (1985)] and root initiation [Malone et al (1978)]. Cadmium also effects the activities of many plant enzymes and it is supposed that this is the first consequence the element has on a plant's metabolic functions [Wiegel and Jager (1980)].

Since the species of tree studied at the Afan 1 forest was Sitka spruce it was decided to attempt a response surface experiment to study the effects of cadmium and copper both singly and in combination, would have upon two years old Sitka spruce trees, such as the levels of pigments and other metals. Response surfaces when used in combination with central composite designs [Bayne and Rubin (1986)] can lend themselves to providing valuable information about the interactions between factors and their respective effects on the system being studied [Morgan (1991)].

The trees were planted in a peat substitute composed of coconut husk and were illuminated using two warm balanced fluorescent tubes, both four feet long. The experiment gave very little results of any statistical significance. One interesting result was that as cadmium levels increased, the length of the needles increased.

By the time the decision was made to end the experiment and to perform the necessary analyses all the trees possessed long spindly needles. Chlorosis was apparent in the older needles of all the trees used in the experiment. Cadmium, due to its toxic nature may be inducing the spindly needles by inhibiting various metabolic pathways within the plant.

The lack of information of any significance may be due to the conditions the trees were grown in. There may have been a lack of illumination of sufficient intensity to promote growth. The peat substitute may have absorbed a large proportion of the nutrients accounting for the observed chlorosis in the older needles. The peat substitute may also have absorbed the cadmium and the copper thus reducing the quantities of these elements going to the plants.

There is still plenty of scope for a follow up on this research project. Atmospheric pollution studies at the Afan 1 forest may yield useful data. The studying of acid fog at the forest could be of importance in relation to acid damage to the foliage and to the possible absorption of organics , such as polyaromatic hydrocarbons (PAH's) into the trees. The use of experimental design methods to determine the effect of 'heavy metals' may have on enzymes in plants could be prove to be useful. Measurements of the throughput of ions through the forest canopy such as been performed in experiments in Germany via the use of model ecosystems [Seufert (1990)] may also prove beneficial.

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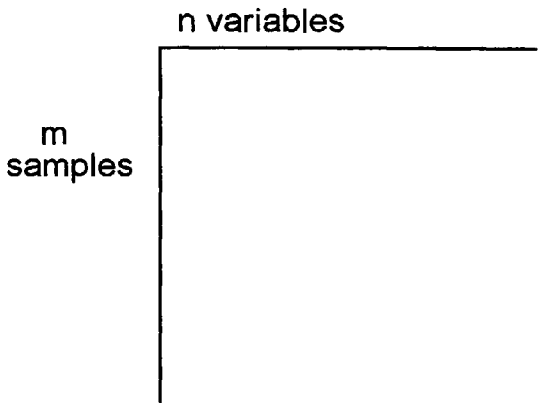
APPENDIX 1.

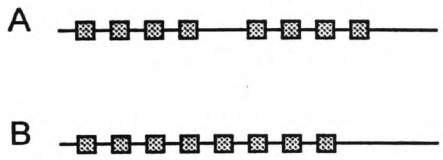
Introduction to Pattern Recognition.

In areas of scientific research, large amounts of data can be produced. These data can be produced from many of the analytical techniques and instrumentation that are becoming quite commonplace in laboratories. Methods to handle these large amounts of data are quite necessary. These techniques generate a multivariate data matrix of the type:



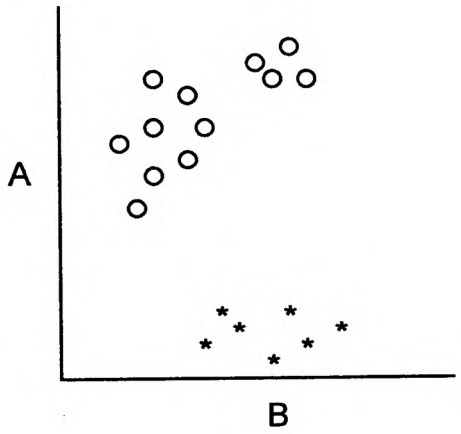
Within this matrix there is information relating to individual samples, relationships between samples and relationships between variables. The problem now is how to extract the useful information from within the data matrix. The normal univariate approach to examining the data by looking at one variable at a time ('eye-balling') is quite inadequate. The multivariate approach must be applied! It is of paramount importance that some chemical knowledge of the system is known in order to define the problem.

Consider the problem where two different measurements (A and B) have been made upon a series of samples with the idea of looking at differences between the samples. This is carried out to determine if the samples are of the same class type. If this was carried out using the univarite method, then one would look at all the A values to see if these could differentiate between the samples, then the same process would be carried out on the B samples e.g.

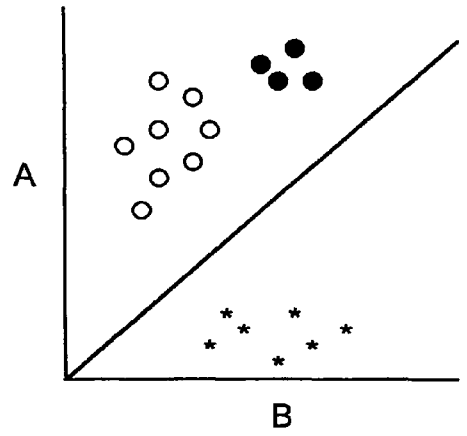


Looking at the A variable it is seen that there is some splitting into two groups, whilst for the B variable there is no split apparent.

With a multivariate approach the values of the A and B variables are used together to examine the objects. This may be achieved by using a bivariate plot (2-dimensional plot) e.g.,



The objects appear from the above diagram to be split into two quite distinct classes. This can be further illustrated if a third variable, C is used to produce a three dimensional plot



Now each object is defined as a point in a three dimensional variable space. Here similarities/ dissimilarities relate to the distances between objects. Many variables can be examined in this way, so that each object becomes a point in n-dimensional space. This becomes more difficult to interpret and this is where multivariate statistical methods come into play, in order to extract the maximum amount of information from within the experimental data being studied.

From the various multivariate (pattern recognition) techniques, it is possible to classify objects on the basis of different measurements (variables), so the any similarity/dissimilarity can be examined.

Classification comes under two main approaches, 'supervised' and 'unsupervised' learning methods. The unsupervised learning method makes no prior assumption about class membership of the objects. This is the approach of exploratory data analysis where one is searching for groups or patterns within the data. With the supervised methods, there is a prior information on the possible class structure of the system being examined. This information can be used to set up rules or strategies by means of which known objects can be classified. Such classification strategies can be checked against a set of test data composed of known objects.

Since objects can be viewed as points in multidimensional variable space, variables can be looked at as points in multidimensional object space (the variables being categorised by the objects). It is therefore possible for the variable to be classified on the basis of similarity or dissimilarity. With pattern recognition, it is often required to produce two dimensional plots of the multivariate data so that relationships between the objects (and groups of objects) can be examined. Here simple bivariate plots will not give sufficient information, since one is looking at the data only using two variables. A better method is the systematic reduction of the whole multivariate data set to produce new 'composite' axes that will retain as much of the information of the objects as possible. Two dimensional or even three dimensional plots using these new axes may be used, thus retaining the information on the significance of the original variables. Basically, a simpler model is being created to describe and understand the system that is understudy. By using a multivariate approach to interpreting the data, the factors that are responsible for the result of the analyses may be determined.

There are now many powerful multivariate statistical packages available. Many of these run on either main frames or personal computers. Some packages have been adapted from mainframe use to run on personal computers. Essentially with this project two main frame packages were used, these being SPSS^{X} (Statistical Package for the Social Sciences) and ARTHUR.

SPSS^{X}

SPSS^{X} was (developed originally at Stanford University in 1965 [Wolff and Parsons (1983)]. This package consists of a number of subroutines, with each one being involved in a different aspect of statistical analysis. SPSS^{X} has its own control language. It has facilities for parametric and non-parametric test, cluster analysis, ANOVA and MANOVA, factor analysis and various forms of correlations. The data must be arranged

in a format useable by SPSS^{X}. The SPSS system is distributed by SPSS Inc., 444 North Michigan Avenue, Chicago, IL 60611.

ARTHUR

This package was developed by Bruce Kowalski at the University of Washington [Wolff and Parsons (1983)] and was then taken over by the Institute of Chemometrics under Kowlaski's direction. The package has many algorithm's and is a very powerful tool. It contains routines for cluster analysis, principal component analysis, KNN (K nearest neighbours), non linear mapping etc... ARTHUR, like SPSS^{X} has its own control language and also like SPSS^{X}, the data must be arranged in an ARTHUR useable format. ARTHUR is distributed by INFOMETRIX ,Inc. P.O. Box 25808, Seattle, WA 98125.

SIMCA

SIMCA Soft Independent Modelling of Class Analogy) was developed by Svante Wold. The package contains a series of routines each of which is menu driven (this makes the package a lot simpler to run compared to the main frame SPSS^{X} and ARTHUR). SIMCA can perform KNN, principal component analysis (PCA), SIMCA is based on the fitting principal components (PC) models separately to separate groups of objects. This allows new objects to be compared to one, several or all of the class models. This means that the object (s) can be assigned to a class model as long as it meets the necessary criteria. SIMCA also contains a routine for performing Partial Least Squares Path Modelling (PLS). This allows the modelling of one or several dependent variables to the rest of the data matrix. PLS is based on disjoint principal component analysis. The PLS method is useful for making predictive models. The SIMCA package is distributed by The Research Group For Chemometrics, Umea Univ., 901 87 UMEA, Sweden, SEPANOVA AB Ostrandsv. 14, s-122 43 ENSKEDE, Sweden.

Cluster Analysis.

Cluster analysis is an autolearning procedure which can provide valuable information on the groupings within a given data set. It can be used for data reduction or simplification; objects which are similar can be grouped together. The cluster analysis for example may also provide unexpected groupings, this can then suggest relationships that may be examined in more detail.

The data involved in a cluster analysis consists of p variables, x_1, x_2,...., x_p for n objects. For hierarchic algorithms these variables are used to produce an array of distances between these objects. The variables are standardised in some way before the distances are determined; this enables all the objects to be equally important. This is ensured by coding, so that all the means are zero and all the variances are one.

There are many different approaches and types of algorithm to perform cluster analysis [Manly (1986)]. The most useful of these are the hierarchic methods, which produce a dendrogram. An important feature associated with cluster analysis is that of distance or similarity. Suppose for example that i and k are two objects of a data set which is to be clustered. It is necessary to quantify their relative positions with respect to one another. Based upon the measurements of the p variables, a distance or similarity between any pair of objects must be calculated. The distance between the objects i and k is normally written as $d_{i,k}$. Many methods exist for the calculation of distance for a set of objects. The most common is the Euclidean distance. In this study cluster the squared Euclidean distance has been used for the cluster analysis..

The distance measure is used by a hierarchical clustering method. In hierarchical methods the distances of each individual from all other individuals. Groups are then formed by a process of of agglomeration or division. With the agglomeration method, all individuals

start off alone in groups of one. Close groups are then gradually merged until finally all individuals are in a single group. With divisive methods, all objects start in a single group. This single group is then split in two groups, and these two groups are then split into further groups until all the objects are in groups of their own [Manly (1986)].

Principal Component Analysis (PCA).

PCA is a transformation of a set of correlated variables, to a new set of uncorrelated variables known as principal components (PC's). These new variables are linear combinations of the original variables and are derived in decreasing order of importance in explaining the variation of the data. This is shown in equation form as:

$$Y_{ik} = \bar{y} + \underbrace{b_1 b_2 \cdots}_{\text{Loadings}} \underbrace{\begin{matrix} t_1 \\ t_2 \end{matrix} x}_{\text{Scores}}$$

With PCA, the data matrix Y is transformed into PC scores and PC loadings (plus error) and each dimension is derived in decreasing order of importance in explaining the variation within the data. The PC scores measure the variation between objects, whilst the PC loadings measure the variation between the variables and in fact provides an explanation of the variation between samples. For each dimension an eigenvalue is also obtained which is a measure of the variation that the dimension explains [Manly (1986)]. PCA is performed on data with n objects and p variables:

Objects	x_1	x_2	. . . .	x_p
1	x_{11}	x_{12}	. . . .	x_{1p}
2	x_{21}	x_{22}	. . . .	x_{2p}
.	.	.	. . . .	.
n	x_{n1}	x_{n2}	. . . .	x_{np}

The first PC is a linear combination of the variables $x_1, x_2 \ldots\ldots x_p$

$$Z_1 = a_{11}x_1 + a_{12}x_2 + \ldots + a_{1p}x_p$$

that varies as much as possible for the individuals subject to the condition that:

$$a_{11}^2 + a_{12}^2 + \ldots + a_{1p}^2 = 1$$

So the variance of Z_1, var (Z_1), is as large as possible given the constraint on the constants a_{ij}. The constant is introduced to stop var (Z_1) being increased just by increasing one of the a_{ij} values. The second PC,

$$Z_2 = a_{21}x_1 + a_{22}x_2 + \ldots + a_{2p}x_p$$

is such that var (Z_2) is as large as possible subject to the constraint that:

$$a_{21}^2 + a_{22}^2 + \ldots + a_{2p}^2 = 1$$

and also to the condition that Z_1 and Z_2 are uncorrelated. For the third PC,....

$$Z_3 = a_{31}x_1 + a_{32}x_2 + \ldots + a_{3p}x_p$$

is such that the var (Z_3) is as large as possible subject to the constraint that:

$$a_{31}^2 + a_{32}^2 + \ldots + a_{3p}^2 = 1$$

and that Z_3 is uncorrelated with Z_1 and Z_2. Further PC's can be extracted in the same manner. If there are p variables than it is possible to have p PC's.

As mentioned earlier PCA involves finding the eigenvalues of the sample covariance matrix. The covariance matrix is symmetrical and is of the form:

$$C = \begin{matrix} C_{11} & C_{13} & \cdots & C_{1p} \\ C_{21} & C_{23} & \cdots & C_{2p} \\ \cdot & \cdot & \cdot & \cdot \\ C_{p1} & C_{p3} & \cdots & C_{pp} \end{matrix}$$

The diagonal component is the variance of x_i and is the covariance of the variables x_i and x_j. The variance of the PC,s are the eigenvalues of the matrix C. There are p of these, some of which may be equal to zero. Negative eigenvalues are not possible for a covariance matrix.

If the eigen values are arranged as $\lambda_1 \geq \lambda_2 \geq \ldots \geq \lambda_p \geq 0$

then corresponds to the ith PC.

$$Z_i = a_{ii} x_1 + a_{12} x_2 + \ldots + a_{ip} x_p$$

Of particular interest, var $(Z_i) = \lambda_i$ and the constants a_{i1}, a_{i2} ... a_{ip} are the elements of the corresponding eigenvalue. The eigenvalues add up to the sum of the diagonal elements of C thus:

$$\lambda_1 + \lambda_2 + \ldots + \lambda_p = C_{11} + C_{22} + \ldots + C_{pp}$$

Since C_{ii} is the variance of x_i and is the variance of Z_i,this means that the sum of the variances of the principal components is equal to the sum of the variances of the original values. Therefore the eigenvalues account for all the variation in the original data set. It is important that no variable has an undue influence on the PC's. In order to achieve this goal, it is necessary to code the variables x_1, x_2, ..., x_p to have means of zero and variances equal to unity at the beginning of the analysis. Matrix C will then have the form:

form:

$$C = \begin{matrix} C_{11} & C_{13} & . & . & C_{1p} \\ C_{21} & 1 & . & . & C_{2p} \\ . & . & . & . & . \\ . & . & . & . & . \\ C_{p1} & C_{p3} & . & . & 1 \end{matrix}$$

Where $C_{ij} = C_{ji}$ is the correlation between x_i and x_j. Thus the PCA is performed on the correlation matrix. Here, the sum of the diagonal terms and the sum of the eigenvalues is equal to p, which is the number of variables.

APPENDIX 2

Grouped Student's t-Test For the 1986 Foliar Analysis, Growth Parameters and Soil-Solution Analysis (mg dm^{-3})

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	4.8340	1.027	.342
Ht					
	Class 1	9	7.3433	2.048	.683

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
2.88	.136	-3.30	17	.004	-3.21	12.68	.007

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.2430	.062	.382
CAI					
	Class 1	9	.6678	.107	..036

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.99	.123	-10.77	17	.000	-10.48	12.43	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.2080	.048	.015
MAI					
	Class 1	9	.3833	.063	.021

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.74	.426	-6.88	17	.000	-6.77	14.88	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	6.8000	1.398	.442
GYC					
	Class 1	9	2.2222	1.202	.401

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.35	.680	-9.01	17	.000	-9.08	16.97	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	3.0000	2.539	.803
PGYC					
	Class 1	9	17.1111	3.621	1.207

		Pooled Variance Estimate		Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	t-value	Degrees of freedom	2-tail Prob.
2.03	.311	-9.92	17	.000	-9.73	14.18

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	2866.6667	513.686	171.229
K yr 3					
	Class 1	9	4401.5000	590.888	208.911

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.32	.699	-5.73	15	.000	-5.68	14.03	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	2870.333	1267.755	422.585
Ca yr 3					
	Class 1	8	2922.150	1227.769	434.082

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.07	.946	-.09	15	.933	-.09	14.87	.933

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	815.000	282.128	94.043
Mg yr 3					
	Class 1	8	501.0000	83.070	29.370

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
11.53	.004	3.02	15	.009	3.19	9.53	.010

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	.6811	.301	.100
Cd yr 3					
	Class 1	8	.6600	.352	.125

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.37	.665	.13	15	.896	.13	13.91	.897

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	4.5822	1.307	.436
Ni yr 3					
	Class 1	8	7.7125	1.278	.452

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.04	.967	-4.98	15	.000	-4.99	14.84	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	3.2978	.698	.233
Cu yr 3					
	Class 1	8	4.5288	.874	.309

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.57	.540	-3.23	15	.006	-3.18	13.41	.007

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	1256.0000	629.277	209.75
Chl a yr 3					
	Class 1	8	1257.25	229.766	81.234

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
7.50	.015	-.01	15	.996	-.01	10.31	.996

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	466.3333	294.238	98.079
Chl b yr 3					
	Class 1	8	445.6250	89.967	31.808

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
10.70	.005	.19	15	.851	.20	9.65	.845

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	9	2.81	.300	.100
a/b yr 3					
	Class 1	8	2.8413	.273	.096

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.21	.816	-.22	15	.826	-.23	14.99	.825

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	3613.9000	916.497	289.822
K yr 2					
	Class 1	9	5328.6667	777.442	259.147

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.39	.654	-4.37	17	.000	-4.41	16.95	.000

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	2226.6000	624.316	197.426
Ca yr 2					
	Class 1	9	3946.1111	2119.585	706.528

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
11.53	.001	-2.26	17	.025	-2.34	9.25	.043

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	758.7000	245.448	77.617
Mg yr 2					
	Class 1	9	723.7778	133.856	44.619

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
3.36	.102	.38	17	.710	.39	14.19	.702

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.5390	.392	.124
Cd yr 2					
	Class 1	9	.4033	.342	.114

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.31	.711	.80	17	.434	.81	16.99	.431

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	3.4310	2.287	.723
Ni yr 2					
	Class 1	9	4.1667	2.155	.718

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.13	.877	-.72	17	.482	-.72	16.95	.480

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	2.1710	1.070	.338
Cu yr 2					
	Class 1	9	2.4789	.663	.221

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.60	.193	-.74	17	.468	-.76	15.21	.458

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	1259.8000	396.285	125.316
Chl a yr 2					
	Class 1	9	1433.4444	444.012	148.004

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.26	.737	-.90	17	.380	-.90	16.19	.384

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	417.6000	164.239	51.937
Chl b yr 2					
	Class 1	9	503.778	149.085	49.695

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.21	.795	-1.19	17	.249	-1.20	17	.247

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	3.1560	.592	.187
a/b yr 2					
	Class 1	9	2.8489	.352	.117

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.83	.158	1.35	17	.194	1.39	14.88	.185

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	4960.4000	544.384	172.384
K yr 1					
	Class 1	9	5752.222	689.305	229.768

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.60	.496	-2.79	17	.012	-2.76	15.24	.014

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	1923.2000	669.492	211.712
Ca yr 1					
	Class 1	9	3229.5556	1541.263	513.754

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
5.30	.022	-2.44	17	.026	-2.35	10.67	.039

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	1082.8000	283.329	89.597
Mg yr 1					
	Class 1	9	1033.778	113.177	37.726

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
6.27	.017	.48	17	.634	.50	12.05	.623

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.7900	.646	.204
Cd yr 1					
	Class 1	9	11.6956	15.721	5.240

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
527.21	.000	-2.08	16	.054	-2.08	8.03	.071

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	4.4530	3.016	.954
Ni yr 1					
	Class 1	9	8.2800	3.915	1.305

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.68	.453	-2.40	17	.028	-2.37	15.02	.032

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	2.3610	1.306	.413
Cu yr 1					
	Class 1	9	2.6467	1.777	.592

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.85	.377	-.40	17	.692	-.40	14.60	.698

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	992.900	363.029	114.800
Chl a yr 1					
	Class 1	9	1078.0000	343.801	81.267

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.22	.276	.-59	17	.561	-.61	15.81	.554

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	308.9000	133.502	42.217
Chl b yr 1					
	Class 1	9	337.5556	80.867	26.956

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.73	.173	-.56	17	.584	-.57	15.02	.576

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	3.3220	.390	.123
a/b yr 1					
	Class 1	9	3.3289	.377	.126

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.07	.933	-.04	17	.969	-.04	16.90	.969

Soil Solution Elemental Analysis

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	1.0308	1.018	.322
Al					
	Class 1	7	1.1003	1.010	.382

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.02	1.000	-.14	15	.891	-.14	13.14	.891

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.1867	.220	.069
As					
	Class 1	7	.0131	.022	.008

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
95.71	.000	2.06	15	.057	2.48	9.27	.034

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.4208	.223	.071
B					
	Class 1	7	.2613	.066	.025

		Pooled Variance Estimate			Separate Variance Estimate		
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
11.47	.008	1.82	15	.089	2.13	11.12	.056

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	4.1800	2.187	.692
Ca					
	Class 1	7	3.8043	3.122	1.180

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.04	.324	.29	15	.773	.27	10.04	.789

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.0067	.009	.003
Cd					
	Class 1	7	.0000	.000	.000

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
.	.	1.96	15	.068	2.37	9.00	.042

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.0474	.016	.005
Cu					
	Class 1	7	.0346	.005	.002

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
10.30	.010	2.09	15	.054	2.44	11.34	.032

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.3455	.354	.112
Fe					
	Class 1	7	.1730	.075	.028

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
22.42	.001	1.26	15	.228	1.49	10.12	.166

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	9.380	9.189	2.906
K					
	Class 1	7	10.6414	10.973	4.148

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.43	.606	-.26	15	.802	-.25	11.49	.809

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	1.6126	.886	.280
Mg					
	Class 1	7	1.2646	.554	.209

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.56	.265	.92	15	.374	.99	14.89	.336

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.1394	.248	.078
Mn					
	Class 1	7	.0294	.026	.010

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
93.95	.000	1.16	15	.265	1.39	9.27	.196

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	11.0040	3.940	1.246
Na					
	Class 1	7	7.5871	1.362	.515

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
8.37	.018	2.19	15	.045	2.53	11.82	.026

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.0175	.015	.005
Ni					
	Class 1	7	.0049	.008	.003

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
3.25	.165	2.01	15	.062	2.22	14.46	.043

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.1820	.230	.073
P					
	Class 1	7	.1366	.178	.067

Pooled Variance Estimate				Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.66	.554	.44	15	.668	.46	14.76	.653

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	6.7670	3.186	1.007
S					
	Class 1	7	4.0757	2.222	.840

Pooled Variance Estimate				Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.06	.393	1.92	15	.074	2.05	14.99	.058

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	2.4700	.758	.240
Si					
	Class 1	7	2.3947	.890	.337

Pooled Variance Estimate				Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
1.38	.636	-.06	15	.952	-.06	11.63	.953

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	.9786	.373	.118
Zn					
	Class 1	7	.6001	.155	.059

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
5.76	.045	2.52	15	.024	2.87	12.84	.013

Variable		No. of Cases	Mean	s.d.	s.e.
	Class 2	10	4.1020	.578	.183
pH					
	Class 1	7	3.7686	.395	.149

	Pooled Variance Estimate			Separate Variance Estimate			
F-value	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.	t-value	Degrees of freedom	2-tail Prob.
2.14	.367	1.32	15	.207	1.41	15.00	.178

APENDIX 3.

Kolmogorov-Smirnov Goodness of Fit Test for Normality for the 1986 Foliar Analyses.

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chla yr3 - Chla yr2 Test distribution - Normal
Mean:.....106.4118
Standard Deviation 571.6921
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.15862	.15862	-.09377	.654	.786

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chla yr3 - Chla yr1 Test distribution - Normal
Mean: 203.5294
Standard Deviation: 419.0331
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.16296	.16296	-.13125	.672	.757

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chla yr2 - Chla yr1
Test distribution - NormalMean: 308.8421
Standard Deviation: 368.8470
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.11100	.11100	-.09671	.484	.973

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chlb yr3 - Chlb yr2
Test distribution - Normal
Mean: -9.8235
Standard Deviation: 216.6357
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.16871	.16871	-.11735	.696	.719

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chlb yr3 - Chlb yr1
Test distribution - Normal
Mean: 123.8235
Standard Deviation: 191.1836
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.24235	.24235	-.15171	.999	.271

----------Kolmogorov - Smirnov Goodness of Fit Test---
Chlb yr2 - Chlb yr1
Test distribution - Normal
Standard Deviation: 137.5308
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.11816	.11816	-.08089	.515	.954

----------Kolmogorov - Smirnov Goodness of Fit Test---
a/b yr3 - a/byr2
Test distribution - NormalMean: -.1988
Standard Deviation: .5036
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.23631	.14965	-.23631	.974	.299

----------Kolmogorov - Smirnov Goodness of Fit Test---
a/b yr3 - a/b yr1
Test distribution - Normal
Standard Deviation: .3674
Mean -.4594
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.18546	.18546	-.16734	.765	.603

----------Kolmogorov - Smirnov Goodness of Fit Test---
a/b yr2 - a/b yr1
Test distribution - Normal
Mean: -.3147
Standard Deviation: .4902
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.16363	.16363	-.09479	.713	.689

----------Kolmogorov - Smirnov Goodness of Fit Test---
K yr3 - Kyr2
Test distribution - Normal
Mean: -864.1177
Standard Deviation: 798.9595
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.09208	.09208	-.08260	.380	.999

----------Kolmogorov - Smirnov Goodness of Fit Test---
K yr3 - K yr6
Test distribution - Normal
Mean: -1730.6471
Standard Deviation: 888.4346
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
14306	.14306	-.10892	.590	.877

----------Kolmogorov - Smirnov Goodness of Fit Test---
K yr2 - K yr1
Test distribution - Normal
Mean: -909.3158
Standard Deviation: 1125.0348
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.12808	.12808	-.10174	.558	.914

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ca yr3 - Ca yr1
Test distribution - Normal
Mean: 213.2353
Standard Deviation: 1282.0427
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.12663	.09959	-.12663	522	.948

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ca yr3 - Ca yr2
Test distribution - Normal
Mean: -290.7059
Standard Deviation: 1593.0557
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.14306	.07994	-.14306	.590	.877

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ca yr2 - Ca yr1
Test distribution - Normal
Mean: 499.1053
Standard Deviation: 633.9191
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.16154	.16154	-.11525	.704	.704

----------Kolmogorov - Smirnov Goodness of Fit Test---
Mg yr3 - Mg yr2
Test distribution - Normal
Mean: -78.8824
Standard Deviation: 316.1924
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.20477	.20477	-.08941	.844	.474

----------Kolmogorov - Smirnov Goodness of Fit Test---
Mg yr3 - Mg yr1
Test distribution - Normal
Mean: -418.2941
Standard Deviation: 228.8006
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.13813	.13813	-.09065	.570	.902

----------Kolmogorov - Smirnov Goodness of Fit Test---
Mg yr2 - Mg yr1
Test distribution - Normal
Mean: -317.4211
Standard Deviation: 228.1051
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.20425	.20425	-.12396	.890	.406

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cd yr3 - Cd yr2
Test distribution - Normal
Mean: .1918
Standard Deviation: .4913
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.18874	.18874	-.12382	.778	.580

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cd yr3 - Cd yr1
Test distribution - Normal
Mean: -5.5500
Standard Deviation: 12.5658
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.44569	.29861	.44569	1.838	.002

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cd yr2 - Cd yr1
Test distribution - Normal
Mean: -6.0856
Standard Deviation: 12.2936
Cases: 18
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.42562	.29637	-.42562	1.806	.003

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ni yr3 - Ni yr2
Test distribution - Normal
Mean: 2.0765
Standard Deviation: 2.7339
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.12917	.12917	-.09231	.533	.939

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ni yr3- Ni yr1
Test distribution - Norma
Mean: -.1147
Standard Deviation: 3.4144
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.23491	.13126	-.23491	.969	.305

----------Kolmogorov - Smirnov Goodness of Fit Test---
Ni yr2 - Ni yr1
Test distribution - Normal
Mean: 2.4863
Standard Deviation: 4.8371
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.13620	.13620	-.08472	.594	.873

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cu yr3 -Cu yr2
Test distribution - Normal
Mean: 1.5718
Standard Deviation: 1.2300
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.19767	.19767	-.08705	.815	.520

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cu yr3 - Cu yr1
Test distribution - Normal
Mean: 1.4276
Standard Deviation: 1.7421
Cases: 17
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.12821	.12821	-.09345	.529	.943

----------Kolmogorov - Smirnov Goodness of Fit Test---
Cu yr2- Cu yr1
Test distribution - Normal
Mean: -.1795
Standard Deviation: 1.7804
Cases: 19
Most extreme differences

Absolute	Positive	Negative	K-S Z	2-Tailed P
.15531	.09545	-.15531	.677	.749

Varible	No. of Cases	Mean	s.d	s.e.
Chl a yr3	17	1256.5882	470.204	114.041
Chl a yr2	17	1363.0000	432.313	104.851

Difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-106.4118	571.692	.200	.442	-.77	16	.454	

Varible	No. of Cases	Mean	s.d	s.e.
Chl a yr3	17	1256.5882	470.204	114.041
Chl a yr1	17	1053.0588	319.363	77.457

Difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
203.5294	419.033	101.630	.491	.045	2.00	16	.062

Varible	No. of Cases	Mean	s.d	s.e.
Chl a yr1	19	1033.2105	306.950	70.419
Chl a yr2	19	1342.0526	417.225	95.718

Dfference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-308.8421	368.847	84.619	.516	.024	-3.65	18	.002

Varible	No. of Cases	Mean	s.d	s.e.
Chl b yr3	17	456.5882	216.663	52.548
Chl b yr2	17	466.4118	164.589	39.919

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-9.8235	216.636	52.542	.380	.132	-.19	16	.854

Varible	No. of Cases	Mean	s.d	s.e.
Chl b yr3	17	456.5882	216.663	52.548
Chl b yr1	17	332.7647	111.665	27.083

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
123.8235	191.184	46.369	.472	.055	2.67	16	.017

Varible	No. of Cases	Mean	s.d	s.e.
Chl b yr2	19	458.4211	159.122	36.505
Chl b yr1	19	322.4737	109.699	25.167

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
135.9474	137.531	31.552	.528	.020	4.31	18	.000

Varible	No. of Cases	Mean	s.d	s.e.
a/b yr3	17	2.8247	.279	.068
a/b yr 1	17	3.2841	.374	.091

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-.4594	.367	.089	.395	.117	-5.16	16	.000

Varible	No. of Cases	Mean	s.d	s.e.
a/b yr2	19	3.0105	.505	.116
a/b yr 1	19	3.3253	.373	.086

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-.3147	.490	.112	.409	.082	-2.80	18	.012

Varible	No. of Cases	Mean	s.d	s.e.
a/b yr3	17	2.8247	.279	.068
a/b yr 2	17	3.0235	.534	.130

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-.3147	.490	.112	.409	.082	-2.80	18	.012

Varible	No. of Cases	Mean	s.d	s.e.
K yr3	17	3588.9412	953.027	231.143
K yr1	17	5319.5882	743.166	180.244

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-1730.6471	888.435	215.477	.474	.055	-8.03	16	.000

Varible	No. of Cases	Mean	s.d	s.e.
K yr3	17	3588.9412	953.027	231.143
K yr2	17	4453.0588	1279.738	310.382

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-864.1176	798.960	193.776	.782	.000	-4.46	16	.000

Varible	No. of Cases	Mean	s.d	s.e.
K yr2	19	4426.1579	1209.301	277.433
K yr1	19	5335.4737	724.118	166.124

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-909.3158	1125.035	258.101	.412	.080	-3.52	18	.002

Varible	No. of Cases	Mean	s.d	s.e.
Ca yr3	17	2894.7059	1209.879	293.439
Ca yr1	17	2681.4706	1320.999	320.389

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
213.2353	1282.043	310.941	.490	.046	.69	16	.503

Varible	No. of Cases	Mean	s.d	s.e.
Ca yr3	17	2894.7059	1209.879	293.439
Ca yr2	17	3185.4118	1752.262	424.986

Difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-290.7059	1593.056	386.373	.471	.056	-.075	16	.463

Varible	No. of Cases	Mean	s.d	s.e.
Ca yr2	19	3041.1053	1723.281	395.348
Ca yr1	19	2542.0000	1314.907	301.660

Difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
499.1053	633.919	145.431	.948	.000	3.43	18	.003

Varible	No. of Cases	Mean	s.d	s.e.
Mg yr3	17	667.2353	262.519	63.670
Mg yr1	17	1085.5294	206.636	50.117

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-418.2941	228.801	55.492	.546	.023	-7.54	16	.000

Varible	No. of Cases	Mean	s.d	s.e.
Mg yr2	19	742.1579	195.976	44.960
Mg yr1	19	1059.5789	215.553	49.451

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-317.4211	228.105	52.331	.389	.100	-6.07	18	.000

Varible	No. of Cases	Mean	s.d	s.e.
Mg yr3	17	667.2353	262.519	63.670
Mg yr2	17	746.1176	206.485	50.080

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-78.8824	316.192	76.688	.107	.683	-1.03	16	.319

Varible	No. of Cases	Mean	s.d	s.e.
Ni yr1	17	6.1700	4.104	.995
Ni yr3	17	6.0553	2.040	.495

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
.1147	3.414	.828	.558	.020	.14	16	.892

Varible	No. of Cases	Mean	s.d	s.e.
Ni yr3	17	6.0553	2.040	.495
Ni yr2	17	3.9788	2.116	.513

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-2.0765	2.734	.663	.135	.605	-3.13	16	.006

Varible	No. of Cases	Mean	s.d	s.e.
Ni yr1	19	6.2658	3.901	.895
Ni yr2	19	3.7795	2.196	.504

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
2.4863	4.837	1.110	-.196	.421	2.24	18	.038

Varible	No. of Cases	Mean	s.d	s.e.
Cd yr2	17	.4794	.387	.094
Cd yr3	17	.6712	.316	.077

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-.1918	.491	.119	.033	.901	-1.62	16	.127

Varible	No. of Cases	Mean	s.d	s.e.
Cu yr1	17	2.4494	1.591	.386
Cu yr3	17	3.8771	.990	.240

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-1.4276	1.742	.423	.151	.562	-3.38	16	.004

Varible	No. of Cases	Mean	s.d	s.e.
Cu yr1	19	2.4963	1.509	.346
Cu yr2	19	2.3168	.891	.204

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
.1795	1.780	.408	-.037	.880	.44	18	.666

Varible	No. of Cases	Mean	s.d	s.e.
Cu yr3	17	3.8771	.990	.240
Cu yr2	17	2.3053	.944	.229

difference mean	s.d.	s.e.	corr.	2-tail Prob.	t-value	degrees of freedom	2-tail Prob.
-1.5718	1.230	.298	.191	.463	-5.27	16	.000

Wilcoxon Matched-Pairs Signed-Ranks Test
Cd yr 3
Cd yr 1

Mean Rank	Cases	
7.29	7	- Ranks (Cd yr 1 LT Cd yr 3)
9.44	9	+ Ranks (Cd yr 1 GT Cd yr 3)
	1	Ties (Cd yr 1 EQ Cd yr 3)
	17 =	Total

Z = -.8790 2-tailed P = .3794

Wilcoxon Matched-Pairs Signed-Ranks Test

Cd yr 2

Cd yr 1

Mean Rank	Cases	
4.20	5	- Ranks (Cd yr 1 LT Cd yr 1)
9.90	9	+ Ranks (Cd yr 1 GT Cd yr 1)
	3	Ties (Cd yr 1 EQ Cd yr 1)
	18 = Total	

Z = -2.2151 2-tailed P = .0268

Appendix 4
Kolmogorov-Smirnov and Student's Paired t-test for the Epicormic and Normal Tissues

Table 1. Kolmogorov-Smirnov Test for Normality in Nutrients and Chlorophyll Levels on Differences Between Normal Tissues and Epicormic Tissues.

			Most Extreme Differences				
Variable	**difference mean**	**sd**	**absolute**	**positive**	**negative**	**K-S Z**	**2-tail Prob.**
Chl a	-65.58	169.66	0.29	0.18	-0.29	1.253	0.087
Chl b	-16.84	48.40	0.25	0.15	-0.25	1.099	0.179
a/b-ratio	0.08	0.25	0.22	0.22	-0.14	0.967	0.307
K	-328.00	1605.63	0.22	0.17	-0.22	0.958	0.318
Ca	-192.95	927. 22	0.10	0.10	-0.10	0.444	0.989
Mg	-121.68	310.03	0.15	0.15	-0.10	0.648	0.795
Cd	-0.42	1.54	0.29	0.23	-0.29	1.273	0.078
Ni	1.32	3.60	0.15	0.09	-0.15	0.650	0.792
Cu	0.95	4.06	0.13	0.13	-0.13	0.561	0.911
Zn	-7.53	21.25	0.09	0.09	-0.09	0.384	0.998

Table 2 Student's Paired t-test for the Epicormic and Normal Tissues E = epicormic

Variable	No. of Cases		Mean		s.d		se
Chl a	19		710.32		142.10		32.60
E Chl a	19		775.90		208.31		47.79
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-65.58	169.58	38.92	0.588	0.008	-1.68	18	0.109
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Chl b	19		199.21		59.80		13.72
E Chl b	19		216.05		69.76		16.00
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-16.84	48.41	11.11	0.731	0.000	-1.52	18	0.147
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
a/b	19		3.30		0.72		0.16
E a/b	19		3.22		0.81		0.19
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
0.08	0.25	0.06	0.954	0.000	1.46	18	0.162
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
K	19		4344.53		1465.58		336.23
E K	19		4672.53		2362.59		542.02
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-328.00	1605.63	368.36	0.744	0.000	-0.89	18	0.385
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Ca	19		1777.89		654.27		150.10
E Ca	19		1970.84		839.79		192.66
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-192.95	927.22	212.72	0.249	0.304	-0.91	18	0.376
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Mg	19		1274.47		328.50		75.36
E Mg	19		1396.16		309.83		71.08
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-121.68	310.03	71.13	0.530	0.020	-1.71	18	0.104
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Cd	19		0.58		1.02		0.23
E Cd	19		1.00		1.63		0.38
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-0.40	0.09	0.35	0.401	0.089	-1.19	18	0.249

Table 2 (Contd.)

Variable	No. of Cases		Mean		s.d		se
Ni	19		6.01		2.94		0.67
E Ni	19		4.70		3.86		0.89
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
1.32	3.60	0.83	0.466	0.044	1.59	18	0.129
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Cu	19		4.68		2.11		0.48
E Cu	19		3.74		3.05		0.70
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
0.95	4.06	0.93	-0.212	0.383	1.02	18	0.323
Variable	**No. of Cases**		**Mean**		**s.d**		**se**
Zn	19		29.20		17.08		3.92
E Zn	19		36.73		20.20		4.64
difference mean	**sd**	**se**	**corr.**	**2-tail Prob.**	**t-value**	**degrees of freedom**	**2-tail Prob.**
-7.53	21.25	4.88	0.360	0.130	-1.54	18	0.140

APPENDIX 5.

ANOVA Tables from the Sitka spruce Seedling Experimental Design.

Chl a/ Chl b ratio (New Growth of Plants)

Run	(Y)	E(Y)	Residuals
1	2.75	2.7276	0.0224
2	2.70	2.8463	-0.1463
3	3.25	3.0952	0.1548
4	2.70	2.7139	-0.0139
5	2.78	2.9184	-0.1384
6	2.88	2.7521	0.1279
7	2.82	2.7208	0.0992
8	2.76	2.8697	-0.1097
9	2.69	2.6912	-0.0012
10	2.76	2.6912	0.0688
11	2.65	2.6912	-0.0412
12	2.96	2.6912	0.2688
13	2.40	2.6912	-0.2912

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	0.4487	12		
Regression	0.1817	5	0.036	0.953
Residual	0.2670	7	0.038	
Lack of fit	0.1035	3	0.034	0.844
Pure Error	0.1635	4	0.041	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b 0	2.691	31.105	b 0	2.806
b 1	-0.059	0.809	b 1	-0.010
b 2	0.066	0.903	b 2	0.005
b 1 1	0.065	0.754	b 1 1	0.000
b 2 2	0.090	1.043	b 2 2	0.000
b 1 2	-0.125	1.280	b 1 2	-0.001

Stationary Point Coordinates
34.6011 x 1 S
17.7204 x 2 S
Response at Staionary Point = 2.67716

Chl b (New Growth of Plants) [x100]

Run	(Y)	E(Y)	Residual
1	6.965	8.0368	-1.0718
2	7.115	7.4041	-0.2891
3	7.269	7.4881	-0.2191
4	8.589	8.0254	0.5636
5	8.469	7.5346	0.9344
6	7.173	7.4742	-0.3012
7	8.830	7.8404	0.9896
8	7.530	7.8864	-0.3564
9	7.423	7.4616	-0.0386
10	7.230	7.4616	-0.2316
11	8.419	7.4616	0.9574
12	6.939	7.4616	-0.5226
13	7.475	7.4616	-0.4146

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	5.7601	12		
Regression	0.6753	5	0.135	0.186
Residual	5.0848	7	0.726	
Lack of fit	3.6681	3	1.223	3.452
Pure Error	1.4168	4	0.354	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b 0	7.462	19.761	b 0	9.296
b 1	-0.018	0.057	b 1	-0.094
b 2	0.024	0.075	b 2	-0.042
b 1 1	0.250	0.667	b 1 1	0.001
b 2 2	0.027	0.071	b 2 2	0.000
b 1 2	0.293	0.686	b 1 2	0.001

Stationary Point Coordinates
27.9770 x 1 S
24.4057 x 2 S
Response at Staionary Point = 7.46639

Carotenoids (New Growth of Plants) [x100]

Run	(Y)	E(Y)	Residual
1	2.950	3.5614	-0.6114
2	2.806	3.2301	-0.4241
3	3.983	3.9326	0.0504
4	4.035	3.7974	0.2376
5	4.072	3.6914	0.3806
6	3.481	3.3959	0.0851
7	3.999	3.2438	0.7552
8	3.549	3.8384	-0.2894
9	3.218	3.1848	0.0332
10	3.204	3.1848	0.0192
11	3.467	3.1848	0.2822
12	3.518	3.1848	0.3332
13	2.333	3.1848	-0.8518

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	3.3528	12		
Regression	1.0163	5	0.203	0.609
Rsidual	1.4188	7	0.334	
Lack of fit	0.9177	3	0.473	2.061
Pure Error	1.4168	4	0.229	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b0	3.185	12.443	b0	4.915
b1	-0.235	1.091	b1	0.542
b2	0.117	0.542	b2	-0.038
b11	0.222	0.222	b11	0.001
b22	0.224	0.224	b22	0.001
b11	0.049	0.049	b11	0.000

Stationary Point Coordinates
38.4605 x 1 S
15.1604 x 2 S
Response at Staionary Point = 3.0998

a/b ratio (Old Growth of Plants)

Run	(Y)	E(Y)	Residual
1	2.62	2.6354	-0.0154
2	2.27	1.8615	0.4085
3	2.50	2.3897	0.1103
4	2.86	2.3257	0.5343
5	3.00	2.9887	0.0113
6	1.80	2.4579	-0.6579
7	1.84	2.0640	-0.2240
8	1.78	2.2025	-0.4225
9	2.97	2.9369	0.0331
10	2.98	2.9369	0.0431
11	3.07	2.9369	0.1331
12	3.14	2.9369	0.2031
13	2.78	2.9369	-0.1569

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	3.0603	12		
Regression	1.8475	5	0.369	2.133
Rsidual	1.2129	7	0.173	
Lack of fit	1.1263	3	0.375	17.357
Pure Error	0.0865	4	0.022	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b0	2.937	15.926	b0	1.001
b1	-0.055	0.352	b1	0.114
b2	0.209	1.351	b2	0.014
b11	-0.501	2.731	b11	-0.002
b22	-0.133	0.726	b22	-0.001
b11	0.178	0.853	b11	0.001

Stationary Point Coordinates
31.4413 x 1 S
32.7551 x 2 S
Response at Staionary Point = 3.02338

Chl a (Old Growth of Plants)

Run	(Y)	E(Y)	Residual
1	1.3847	1.4839	-0.0992
2	1.4222	1.3875	0.0353
3	1.4086	1.4015	0.0071
4	1.5031	1.3615	0.1416
5	1.6011	1.5214	0.0797
6	1.3024	1.4350	-0.1326
7	1.5604	1.5029	0.0575
8	1.3238	1.4342	-0.1104
9	2.0288	1.7366	0.2922
10	1.7505	1.7366	0.0139
11	1.9103	1.7366	0.1737
12	1.6882	1.7366	-0.0484
13	1.3259	1.7366	-0.4107

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	0.6492	12		
Regression	0.2918	5	0.058	1.143
Rsidual	0.3574	7	0.051	
Lack of fit	0.0706	3	0.024	0.328
Pure Error	0.2868	4	0.072	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b0	1.737	17.348	b0	0.720
b1	0.027	0.322	b1	0.045
b2	0.034	0.405	b2	0.029
b11	-0.167	1.678	b11	-0.001
b22	-0.161	1.617	b22	-0.001
b11	0.014	0.125	b11	0.000

Stationary Point Coordinates
31.2875 x 1 S
21.6449 x 2 S
Response at Staionary Point = 1.73960

Chl b (Old Growth of Plants)

Run	(Y)	E(Y)	Residual
1	5.267	5.8152	-0.5482
2	6.261	7.3359	-1.0749
3	5.643	5.9676	-0.3246
4	5.251	6.1023	-0.8513
5	5.333	4.8767	0.4563
6	7.213	5.9254	1.2876
7	8.461	7.4125	1.0485
8	7.423	6.7276	0.6954
9	6.829	5.9524	0.8766
10	5.878	5.9524	-0.0744
11	6.230	5.9524	0.2776
12	5.375	5.9524	-0.5774
13	4.761	5.9524	-1.1914

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	13.6873	12		
Regression	5.3485	5	1.070	0.898
Rsidual	8.3388	7	1.191	
Lack of fit	5.7350	3	1.912	2.937
Pure Error	2.6038	4	0.651	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b0	5.952	12.310	b0	7.213
b1	0.270	0.655	b1	-0.137
b2	-0.414	1.018	b2	0.080
b11	0.696	1.448	b11	0.003
b22	-0.344	0.715	b22	-0.002
b11	-0.346	0.635	b11	-0.002

Stationary Point Coordinates
25.4111 x 1 S
13.2748 x 2 S
Response at Staionary Point = 6.00374

Carotenoids (Old Growth of Plants)

Run	(Y)	E(Y)	Residual
1	2.808	3.2548	-0.4468
2	2.723	4.0443	-1.3213
3	4.330	3.8530	0.4770
4	2.678	3.0755	-0.3975
5	3.156	3.3202	-0.1642
6	4.544	3.3278	1.2162
7	5.154	3.8988	1.2552
8	3.461	3.6641	-0.2031
9	3.384	3.5357	-0.1517
10	3.382	3.5357	-0.1537
11	3.676	3.5357	0.1403
12	3.282	3.5357	-0.2537
13	3.539	3.5357	0.0033

Analysis of Variance

Source	Sums of Squares	DF	Mean Squares	F Ratio
Total	6.4708	12		
Regression	0.8863	5	0.177	0.222
Rsidual	5.5845	7	0.798	
Lack of fit	5.4538	3	1.818	55.634
Pure Error	0.1307	4	0.033	

Coded Coefficients			Uncoded Coefficients	
Coefficient	Estimate	t-Statistic	Coefficient	Estimate
b0	3.536	8.935	b0	2.686
b1	0.093	0.279	b1	0.000
b2	-0.003	0.009	b2	0.075
b11	0.153	0.389	b11	0.001
b22	-0.132	0.335	b22	-0.001
b11	-0.392	0.877	b11	-0.002

Stationary Point Coordinates
28.3557 x 1 S
22.2739 x 2 S
Response at Staionary Point = 3.53036

% Increase in tree height [x10]

Run	(Y)	E(Y)	Residuals
1	4.51	4.1830	0.3270
2	6.09	4.8326	1.2574
3	4.85	5.8843	-1.0343
4	5.05	5.1538	-0.1038
5	5.95	5.3547	0.5953
6	4.43	5.3034	-0.8734
7	3.57	4.7834	-1.2134
8	7.00	6.0647	0.9353
9	6.97	6.8520	0.1180
10	5.8	6.8520	-1.0520
11	7.55	6.8520	0.6980
12	6.39	6.8520	-0.4620
13	7.66	6.8520	0.8080

Analysis of Variance:

Source	Sums of Squares	d.f	Mean Squares	F ratio
Total	19.7510	12		
Regression	11.0439	5	2.209	1.776
Residual	8.7072	7	1.244	
Lack of fit	6.2330	3	2.078	3.359
Pure error	2.4741	4	0.619	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	6.852	13.867	b 0	1.669
b 1	-0.506	1.217	b 1	0.234
b 2	0.020	0.049	b 2	0.216
b 1 1	-0.890	1.811	b 1 1	-0.004
b 2 2	-0.949	1.931	b 2 2	-0.004
b 1 2	-0.345	0.619	b 1 2	-0.002

Stat point coordinates
25.5531 x 1S
20.9685 x 2 S
Response at Stationary Point = 6.92759

fresh weight apical shoot [x10]

Run	(Y)	E(Y)	Residual
1	0.70	0.6738	0.0262
2	0.64	0.8061	-0.1661
3	1.15	0.8782	0.2718
4	0.93	0.8505	0.0795
5	0.74	0.9577	-0.2177
6	1.11	1.0240	0.0860
7	0.66	0.5320	0.1280
8	0.43	0.6897	-0.2597
9	0.81	0.7956	0.0144
10	0.51	0.7956	-0.2856
11	1.12	0.7956	0.3244
12	0.85	0.7956	0.0544
13	0.74	0.7956	-0.0556

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	0.6239	12		
Regression	0.1838	5	0.037	0.585
Residual	0.4401	7	0.063	
Lack of fit	0.2471	3	0.082	1.706
Pure error	0.1931	4	0.048	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	0.796	7.162	b 0	0.604
b 1	0.062	0.666	b 1	0.030
b 2	0.026	0.280	b 2	0.018
b 1 1	0.115	1.042	b 1 1	0.001
b 2 2	0.122	1.101	b 2 2	0.001
b 1 2	0.040	0.319	b 1 2	0.000

Stationary point coordinates
25.7883 x 1 S
20.9217 x 2 S
Response at Stationary Point = 0.80352

Dry Weight/Fresh Weight Ratio of Needles

Run	(Y)	E(Y)	Residuals
1	7.20	7.4964	-0.2964
2	6.11	6.3480	-0.2380
3	7.49	7.3841	0.1059
4	7.69	7.5257	0.1643
5	7.50	7.3716	0.1284
6	6.77	6.7338	0.0362
7	7.56	7.1602	0.3998
8	7.60	7.8352	-0.2352
9	7.83	7.6270	0.2030
10	7.76	7.6270	0.1330
11	7.49	7.6270	-0.1370
12	7.53	7.6270	-0.0970
13	7.46	7.6270	-0.1670

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	2.6085	12		
Regression	2.0779	5	0.416	5.482
Residual	0.5306	7	0.076	
Lack of fit	0.4157	3	0.139	4.821
Pure error	0.1150	4	0.029	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	7.627	62.529	b 0	7.727
b 1	-0.266	2.598	b 1	-0.025
b 2	0.252	2.455	b 2	0.037
b 1 1	-0.081	0.664	b 1 1	-0.000
b 2 2	-0.358	2.951	b 2 2	-0.002
b 1 2	0.323	2.343	b 1 2	0.001

Stationary point coordinates coded
-118.704 x 1 S
-41.7177 x 2 S
Response at Stationary Point = 8.430558

Calcium levels in the roots [x100]

Run	E(Y)	E(Y)	Residual
1	10.27	9.7616	0.5084
2	9.400	8.5138	0.8862
3	5.338	5.7639	-0.4259
4	8.083	8.1312	-0.0482
5	7.475	7.4636	0.0114
6	7.588	8.1729	-0.5849
7	8.545	9.5692	-1.0242
8	7.245	6.7943	0.4507
9	7.420	7.8269	-0.4069
10	8.108	7.8269	0.2811
11	7.150	7.8269	-0.6769
12	7.095	7.8269	-0.7319
13	9.588	7.8269	1.7611

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	19.8926	12		
Regression	12.7308	5	2.546	2.489
Residual	7.1618	7	1.023	
Lack of fit	2.8219	3	0.941	0.867
Pure error	4.3399	4	1.085	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	7.827	17.466	b 0	9.298
b 1	1.095	2.907	b 1	-0.066
b 2	-0.280	0.743	b 2	-0.138
b 1 1	0.221	0.496	b 1 1	0.001
b 2 2	-0.005	0.012	b 2 2	-0.000
b 1 2	0.904	1.787	b 1 2	0.004

Stat point coordinates coded
34.3887 x 1 S
-0.3196 x 2 S
Response at Stationary Point = 8.17666

Copper levels in the roots

Run	(Y)	E(Y)	Residual
1	11.7	12.8659	-1.1659
2	9.9	10.0162	-0.1162
3	8.6	9.1545	-0.5545
4	11.8	11.3048	0.4952
5	9.9	8.6536	1.2464
6	7.8	8.2106	-0.4106
7	14.5	13.5995	0.9005
8	12	12.0647	-0.0647
9	8.1	9.8060	-1.7060
10	9.3	9.8060	-0.5060
11	9.5	9.8060	-0.3060
12	7.7	9.8060	-2.1060
13	14.1	9.8060	4.2940

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Square	F ratio
Total	61.8031	12		
Regression	31.2066	5	6.241	1.428
Residual	30.5965	7	4.371	
Lack of fit	4.4627	3	1.488	0.228
Pure error	26.1338	4	6.533	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	9.806	10.587	b	17.719
b 1	0.606	0.778	b 1	-0.574
b 2	0.175	0.225	b 2	-0.003
b 1 1	1.885	2.047	b 1 1	0.008
b 2 2	-0.856	0.930	b 2 2	-0.004
b 1 2	1.250	1.196	b 1 2	0.006

Statioary point coordinates coded
27.6545 x 1 S
19.8194 x 2 S
Response at Stationary Point = 9.75778

Cd levels in the shoot

Run	(Y)	E(Y)	Residuals
1	0.068	-0.0628	0.1308
2	0.247	0.3775	-0.1305
3	1.294	0.9266	0.3674
4	0.169	0.0629	0.1061
5	0.081	0.4347	-0.3539
6	0.225	0.1664	0.0586
7	0.206	0.1669	0.0391
8	0.260	0.5944	-0.3344
9	0.684	0.3999	0.2841
10	0.232	0.3999	-0.1679
11	0.773	0.3999	0.3731
12	0.190	0.3999	-0.2099
13	0.237	0.3999	-0.1629

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Square	F ratio
Total	1.4727	12		
Regression	0.7316	5	0.146	1.382
Residual	0.7411	7	0.106	
Lack of fit	0.4224	3	0.141	1.767
Pure error	0.3187	4	0.080	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	0.400	2.774	b 0	-0.432
b 1	-0.169	1.392	b 1	0.021
b 2	0.106	0.874	b 2	0.062
b 1 1	-0.012	0.084	b 1 1	-0.000
b 2 2	-0.062	0.432	b 2 2	-0.000
b 1 2	-0.326	2.004	b 1 2	-0.001

Stat point coordinates coded
38.0361 x 1 S
11.6449 x 2 S
Response at Stationary Point = 0.32523

Fe levels shoot [x10]

Run	(Y)	E(Y)	Residuals
1	2.855	4.7998	-1.9448
2	2.436	2.3655	0.0705
3	2.464	4.5148	-2.0508
4	3.934	3.9694	-0.0354
5	8.132	5.3075	2.8245
6	3.063	3.4198	-0.3568
7	3.645	2.4944	1.1502
8	4.674	3.3300	1.3174
9	2.314	2.5227	-0.2087
10	2.641	2.5227	0.1183
11	1.768	2.5227	-0.7547
12	3.034	2.5227	0.5113
13	1.882	2.5227	-0.6407

Analysis of Variance:

Source	Sums of Squares	d.f	Mean Squares	F ratio
Total	33.2184	12		
Regression	12.7612	5	2.552	0.873
Residual	20.4572	7	2.922	
Lack of fit	19.1581	3	6.386	19.662
Pure error	1.29914	0.325		

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	2.523	3.331	b 0	6.460
b 1	-0.330	0.518	b 1	-0.129
b 2	0.745	1.170	b 2	-0.217
b 1 1	0.243	0.322	b 1 1	0.001
b 2 2	1.147	1.523	b 2 2	0.005
b 1 2	0.472	0.552	b 1 2	0.002

Stat point coordinates coded
48.6496 x 1 S
11.2875 x 2 S
Response at Stationary Point = 2.101256

Fe levels root [x100]

Run	(Y)	E(Y)	Residuals
1	0.7855	1.1473	-0.3617
2	1.2779	1.5400	-0.2620
3	0.8943	0.9995	-0.1052
4	1.0506	1.0561	-0.0055
5	0.9589	0.6515	0.3075
6	1.0862	0.9361	0.1501
7	2.1717	1.7404	0.4313
8	1.3665	1.3402	0.0263
9	1.4434	1.0912	0.3521
10	0.4307	1.0912	-0.6605
11	1.0181	1.0912	-0.0732
12	1.2689	1.0912	0.1776
13	1.1445	1.0912	0.0232

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	1.9915	12		
Regression	0.8794	5	0.176	1.107
Residual	1.1121	7	0.159	
Lack of fit	0.5144	3	0.171	1.147
Pure error	0.5977	4	0.149	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	Statatistic	Coefficients	Estimate
b 0	1.091	6.180	b 0	1.490
b 1	0.158	1.064	b 1	-0.057
b 2	-0.112	0.757	b 2	0.037
b 1 1	0.280	1.594	b 1 1	0.001
b 2 2	-0.185	1.056	b 2 2	-0.001
b 1 2	-0.084	0.422	b 1 2	-0.000

Stat point coordinates coded
25.2431 x 1 S
16.5317 x 2 S
Response at Stationary Point = 1.07918

Mg levels in the shoots [x10]

Run	(Y)	E(Y)	Residuals
1	9.84	10.8295	-0.9895
2	2.64	8.2430	-5.6030
3	7.29	7.3363	-0.0463
4	12.41	17.0698	-4.6598
5	11.14	11.2614	-0.1214
6	22.95	15.7890	7.1610
7	10.59	6.3258	4.2642
8	12.48	9.7046	2.7754
9	10.27	10.3661	-0.0961
10	12.03	10.3661	1.6639
11	9.72	10.3661	-0.6461
12	7.68	10.3661	-2.6861
13	9.35	10.3661	-1.0161

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	247.5103	12		
Regression	104.7981	5	20.960	1.028
Residual	142.7122	7	20.387	
Lack of fit	131.2693	3	43.756	15.295
Pure error	11.4430	4	2.861	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	0.366	5.182	b 0	21.280
b 1	-1.333	0.793	b 1	0.027
b 2	-1.787	1.063	b 2	-0.879
b 1 1	-1.465	0.736	b 1 1	-0.006
b 2 2	1.968	0.990	b 2 2	0.009
b 1 2	3.080	.364	b 1 2	0.014

Stat point coordinates coded
30.1791 x 1 S
26.6731 x 2 S
Response at Stationary Point = 9.96097

Mg levels root [x10]

Run	(Y)	E(Y)	Residuals
1	1.5310	1.5443	-0.0133
2	1.6468	1.6158	0.0310
3	1.1236	1.1823	-0.0587
4	1.1761	1.1905	-0.0144
5	1.1743	1.1220	0.0523
6	1.1549	1.1725	-0.0176
7	1.8166	1.8351	-0.0185
8	1.3895	1.3364	0.0531
9	1.6230	1.2985	0.3245
10	1.0581	1.2985	-0.2404
11	1.0109	1.2985	-0.2876
12	1.3294	1.2985	0.0309
13	1.4573	1.2985	0.1588

Analysis of Variance:

Source	Sums of Squares	d.f	Mean Squares	F ratio
Total	0.7770	12		
Regression	0.4940	5	0.099	2.444
Residual	0.2830	7	0.040	
Lack of fit	0.0110	3	0.004	0.054
Pure error	0.2720	4	0.068	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	1.298	14.577	b 0	1.438
b 1	0.197	2.629	b 1	-0.033
b 2	-0.020	0.266	b 2	0.018
b 1 1	0.179	2.021	b 1 1	0.001
b 2 2	-0.094	1.063	b 2 2	-0.000
b 1 2	-0.016	0.157	b 1 2	-0.000

Stat point coordinates coded
21.7111 x 1 S
19.1090 x 2 S
Response at Stationary Point = 1.24468

Na levels root [x100]

Run	(Y)	E(Y)	Residuals
1	8.448	7.4463	1.0017
2	4.320	5.1197	-0.7997
3	2.118	2.0370	0.0810
4	3.463	5.1834	-1.7204
5	3.320	4.2940	-0.9740
6	6.683	4.8134	1.8696
7	6.198	6.4769	-0.2789
8	4.265	3.0905	1.1745
9	1.055	3.5396	-2.4846
10	2.625	3.5396	-0.9246
11	4.505	3.5396	0.9584
12	2.948	3.5396	-0.5916
13	6.228	3.5396	2.6884

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	51.8591	12		
Regression	25.8242	5	5.165	1.389
Residual	26.0349	7	3.719	
Lack of fit	10.5108	3	3.504	0.903
Pure error	15.5241	4	3.881	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	3.540	4.143	b 0	9.017
b 1	1.336	1.861	b 1	-0.240
b 2	-0.205	0.285	b 2	-0.308
b 1 1	0.775	0.912	b 1 1	0.003
b 2 2	0.632	0.744	b 2 2	0.003
b 1 2	1.368	1.419	b 1 2	0.006

Stat point coordinates coded
-305.86146 x 1 S
386.3925 x 2 S
Response at Stationary Point = -13.91518

K levels shoot [x1000]

Run	(Y)	E(Y)	Residual
1	2.2076	2.0102	0.1974
2	0.8820	0.0480	0.8339
3	1.7542	2.0064	-0.2522
4	2.1896	1.8053	0.3843
5	2.5820	2.4420	0.1399
6	0.2068	1.0716	-0.8648
7	0.1986	0.9159	-0.7173
8	2.0192	2.0267	-0.0076
9	2.1765	2.2099	-0.0334
10	2.7655	2.2099	0.5556
11	2.0866	2.2099	-0.1233
12	2.0819	2.2099	-0.1280
13	2.2253	2.2099	0.0154

Analysis of Variance:

Source	Sums of Squares	d.f	Mean Squares	F ratio
Total	8.3414	12		
Regression	5.7719	5	1.154	3.145
Residual	2.5695	7	0.367	
Lack of fit	2.2278	3	0.743	8.694
Pure error	0.3417	4	0.085	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	2.210	8.233	b 0	1.199
b 1	-0.438	1.943	b 1	0.054
b 2	0.541	b 2	2.397	0.028
b 1 1	-0.460	1.724	b 1 1	-0.002
b 2 2	-0.282	1.058	b 2 2	-0.001
b 1 2	0.440	1.453	b 1 2	0.002

Stat point coordinates coded
29.5548 x 1 S
34.0141 x 2 S
Response at Stationary Point = 2.46908

Mn levels shoot [x10]

Run	(Y)	E(Y)	Residuals
1	8.849	8.5235	0.3255
2	3.216	3.5530	-0.3370
3	4.840	8.2834	-3.4434
4	8.708	12.8139	-4.1059
5	7.593	5.7605	1.8325
6	8.360	5.4817	2.8783
7	6.724	7.3433	-0.6193
8	18.388	13.0579	5.3301
9	5.538	6.3562	-0.8182
10	5.942	6.3562	-0.4142
11	7.090	6.3562	0.7338
12	4.507	6.3562	-1.8492
13	6.843	6.3562	0.4868

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	164.3783	12		
Regression	89.9716	5	17.994	1.693
Residual	74.4067	7	10.630	
Lack of fit	69.3709	3	23.124	18.367
Pure error	5.0358	4	1.259	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	6.356	4.401	b 0	25.829
b 1	-2.255	1.857	b 1	-1.001
b 2	0.110	0.091	b 2	-0.228
b 1 1	2.395	1.668	b 1 1	0.011
b 2 2	-0.458	0.319	b 2 2	-0.002
b 1 2	2.375	1.457	b 1 2	0.011

Stat point coordinates coded
32.7030 x 1 S
28.7966 x 2 S
Response at Stationary Point = 6.18526

K levels root [x1000]

Run	(Y)	E(Y)	Residuals
1	2.8670	2.5576	0.3094
2	2.2505	2.0832	0.1673
3	1.8785	1.7116	0.1669
4	2.2130	2.1882	0.0248
5	1.7528	2.0732	-0.3204
6	1.9785	2.0746	-0.0961
7	2.0303	2.3510	-0.3207
8	1.7858	1.8816	-0.0958
9	1.5023	1.9327	-0.4304
10	2.2795	1.9327	0.3468
11	1.7920	1.9327	-0.1407
12	1.6768	1.9327	-0.2559
13	2.5775	1.9327	0.6448

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	1.7637	12		
Regression	0.5809	5	0.116	0.688
Residual	1.1828	7	0.169	
Lack of fit	0.3762	3	0.125	0.622
Pure error	0.8066	4	0.202	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	1.933	10.613	b 0	2.812
b 1	0.185	1.210	b 1	-0.039
b 2	-0.001	0.004	b 2	-0.047
b 1 1	0.114	0.632	b 1 1	0.001
b 2 2	0.088	0.486	b 2 2	0.000
b 1 2	0.238	1.157	b 1 2	0.001

Stat point coordinates coded
60.1643 x 1 S
-20.7509 x 2 S
Response at Stationary Point = 2.11959

Mn levels root [x10]

Run	(Y)	E(Y)	Residuals
1	2.495	2.0306	0.4644
2	1.138	1.0266	0.1114
3	1.036	0.8582	0.1778
4	0.855	1.0302	-0.1752
5	0.900	1.3587	-0.4587
6	0.930	0.8316	0.0984
7	1.274	1.6804	-0.4064
8	0.986	0.9400	0.0460
9	1.106	1.0655	0.0405
10	0.899	1.0655	-0.1665
11	1.315	1.0655	0.2495
12	1.197	1.0655	0.1315
13	0.953	1.0655	-0.1125

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	2.1942	12		
Regression	1.3949	5	0.279	2.443
Residual	0.7993	7	0.114	
Lack of fit	0.6777	3	0.226	7.434
Pure error	0.1216	4	0.030	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	1.066	7.118	b 0	1.631
b 1	0.292	2.322	b 1	-0.047
b 2	0.208	1.653	b 2	-0.029
b 1 1	0.152	1.024	b 1 1	0.001
b 2 2	0.018	0.124	b 2 20.000	
b 1 2	0.294	1.740	b 1 2	0.001

Stat point coordinates coded
19.9429 x 1 S
15.5432 x 2 S
Response at Stationary Point = 0.93676

Ca levels shoot [x1000]

Run	(Y)	E(Y)	Residuals
1	2.2108	2.3994	-0.1886
2	1.5013	1.5316	-0.030
3	1.9688	2.1015	-0.1327
4	2.5595	2.5339	0.0256
5	2.6945	2.4680	0.2265
6	2.1688	2.1922	-0.0234
7	2.0063	1.8607	0.1456
8	2.3645	2.3070	0.0575
9	2.5200	2.4724	0.0476
10	2.8550	2.4724	0.3826
11	2.2375	2.4724	-0.2349
12	2.1050	2.4724	-0.3674
13	2.5645	2.4724	0.0921

Analysis of Variance:

Source	Sums of Squares	d.f.	Mean Squares	F ratio
Total	1.5529	12		
Regression	1.0745	5	0.215	3.144
Residual	0.4784	7	0.068	
Lack of fit	0.1311	3	0.044	0.503
Pure error	0.3473	4	0.087	

Coded Coefficents			Uncoded Coefficients	
Coefficient	Estimate	t Statatistic	Coefficients	Estimate
b 0	2.472	21.347	b 0	2.421
b 1	-0.176	1.809	b 1	0.024
b 2	.118	0.1091	b 2	-0.020
b 1 1	-0.242	2.103	b 1 1	-0.001
b 2 2	-0.089	0.770	b 2 2	-0.000
b 1 2	0.325	2.487	b 1 2	0.001

Stat point coordinates coded
26.9153 x 1 S
23.5463 x 2 S
Response at Stationary Point = 2.50348

APPENDIX 6.

Published Work.

Organizing Committee

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The analysis of chlorophylls in plant extracts using packed column SFC

by

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There is widespread evidence of the decline of forest vitality in many areas of Europe and North America. Amongst others, two of the symptoms of damage to coniferous forests are defoliation and chlorosis, the cause of which is attributed to atmospheric pollution (i.e. acid rain). It is becoming increasingly apparent that the amount of certain pigments within plants (carotenoids, chlorophylls) may be used as a diagnostic parameter for plant condition monitoring.

The aim of this study, under sponsorship of the UK Forestry Commission is to investigate the applicability of SFE and packed column SFC for the routine quantitative analysis of chlorophyll 'a' and 'b' in Sitka spruce (*Picea sitchensis*). Plant extract samples were prepared using a procedure of homogenisation, filtration and "dioxane complex" precipitation. Pure samples of chlorophyll 'a' and 'b' were either purchased or prepared by preparative liquid chromatography using a sucrose column.

Our studies have centred on the optimization of the separation of chlorophyll 'a' from 'b' within a plant extract. Packed column SFC using an amine column (250 mm x 4.6 mm) with modified carbon dioxide at 100 °C utilizing UV detection at 436 nm produced good resolution (fig. 1). The sensitivity of the method under these conditions was excellent giving a detection limit for chlorophyll 'a' of 1.8 ng injected on column (180 g/1000 cm^3 of plant extract) for a signal / noise ratio of 3:1 (fig. 2). Investigations into the quantitative aspects of the method are promising. A sequence of standard injections over the range 10 mg to 200 mg/1000 cm^3 gave a non linear peak area response. However, the method is reproducible, giving a percentage standard deviation of 1.5 % for a sequence of consecutive injections of the same concentration. Currently studies are continuing in order to improve the method by utilizing an appropriate internal standard.

We will discuss our initial findings and hope to be able to report results of ongoing investigations into quantitative aspects of the method.

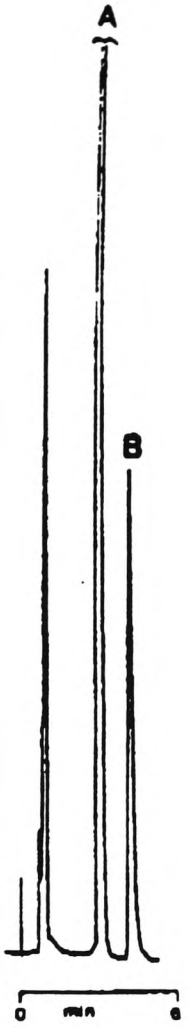

Fig. 1. Packed column SFC separation (A) Chlorophyll 'a', (B) Chlorophyll 'b'.
Column - 5 μ NH_2 Alphasil 250 mm x 4.6 mm. Oven temperature - 130 °C. Pressure - 3500 psi. Flow - 5 cm^3 min^{-1}. Mobile phase - carbon dioxide / methoxyethanol (3% formaldehyde). 80:20. Detection UV @ 436 nm.

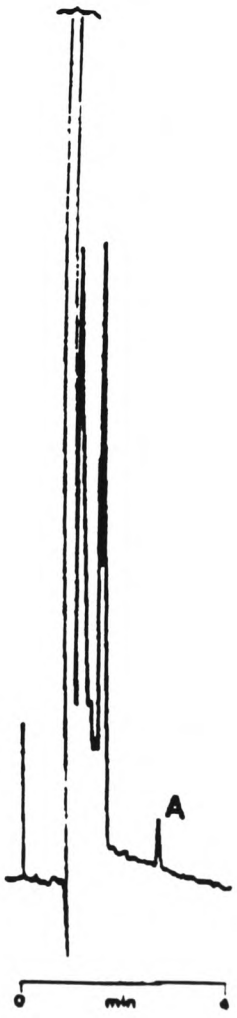

Fig. 2. Detection of chlorophyll 'a' (A), (1.8 ng).
Conditions as figure 1.

IONOPHORES: POTENTIAL SCREENING VIA SUPERCRITICAL FLUID CHROMATOGRAPHY COMBINED WITH MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY

by

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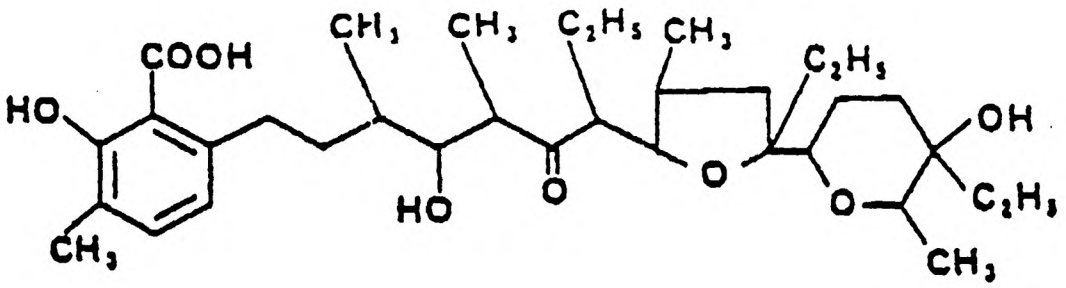
Summary

Lasalocid, monensin, salinomycin and narasin are members to a group of carboxylic polyether antibiotics which possess ionophoric properties. This group of compounds have found application as veterinary drugs since they possess antimicrobial activity[1] and are currently used by poultry and beef farmers. These compounds, normally in the form of their sodium salts, are typically added at levels ranging between 60 to 100 mg kg^{-1} of medicated animal feeds. Above the recommended dosage level concern arises due to the toxicity of the ionophores or their administration to species of animals for which they were not intended. Thus for survelliance and enforcement purposes a necessity arises for the sensitive monitoring of individual ionophores in animal feeds.

Published methods for the determinaton of ionophores include; colorimetric reaction[2], thin layer chromatography (TLC)[3], bioautography[4], normal phase high performance liquid chromatography (HPLC)[5], reverse phase HPLC[6] and a procedure involving confirmation of results by fast atom bombardment mass spectrometry (MS) following TLC analysis[7].

Packed column supercritical fluid chromatography (SFC) offers advantages over conventional HPLC in terms of speed of analysis, rapidity of method development and superior separation efficiency with unit time. An intrinsic problem associated with HPLC analysis of ionophores is the method of detection. Members of this class of compounds do not possess strong chromophores, thus methods involving post column derivatisation have been developed to facilitate the more effective use of ultraviolet detection[5,6].

We now report the potential of SFC combined with light scattering mass detection as a rapid means of analysing underivatised ionophores. Confirmation of the results obtained for lasalocid is achieved via SFC/MS and SFC/MS/MS. The latter technique is likely to be of greatest value where minimal sample workup procedures are to be implemented for the analysis of complex biological matricies.



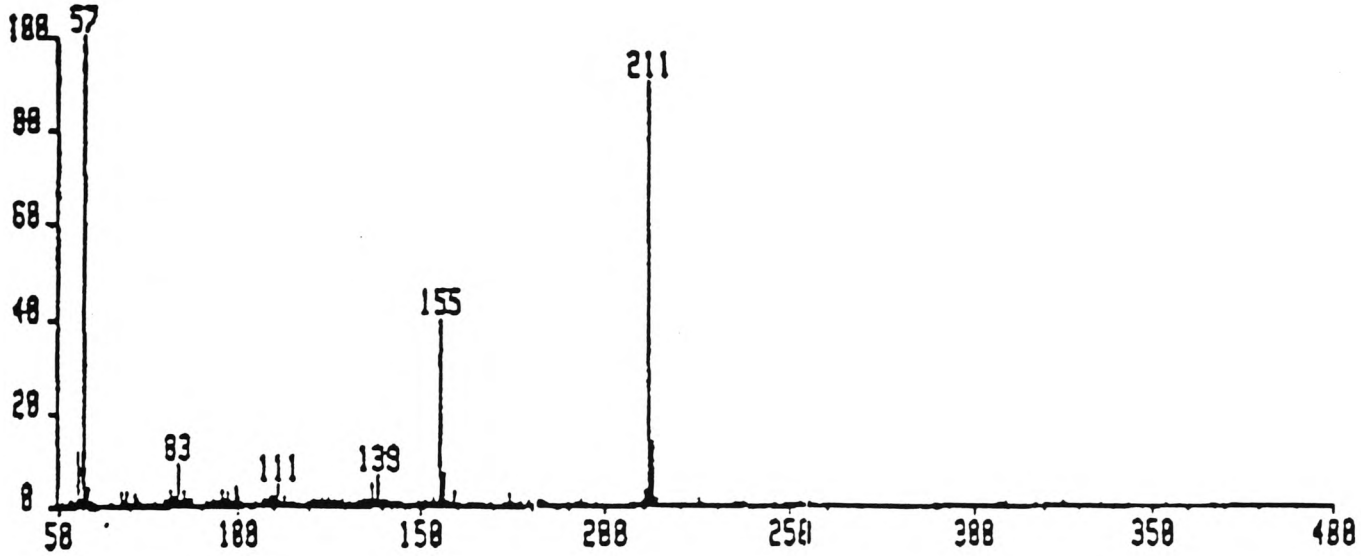


Figure 1. SFC/MS mass spectra of lasalocid obtained by EI.

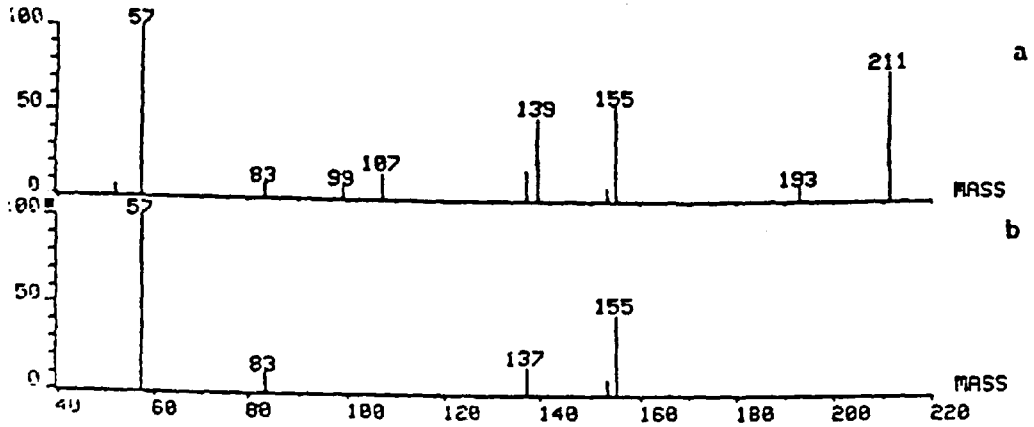


Figure 2. CID daughter ion spectra recorded at 16 eV collision energy for EI primary ions.
(a) m/z 211 and (b) m/z 155.

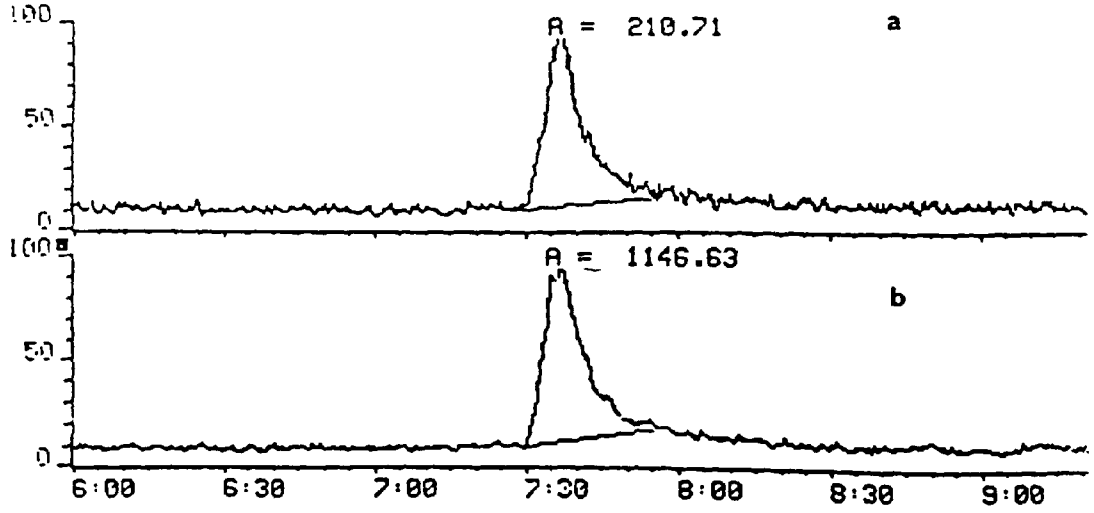


Figure 3. MRM chromatograms obtained for 10 ng lasalocid injected on - column.
(a) m/z 211-57 and (b) m/z 155-57.

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APPENDIX 7

Map References for the Afan 1 Forest Sites

Site No	Grid Reference	Soil Type	Elevation (m)
1	940 026	peaty gley	470
2	935 026	peaty gley	460
3	939 019	peaty gley	440
4	906 017	deep peat	515
5	902 994	peaty iron-pan	500
6	902 993	deep peat	500
7	907 983	peaty gley	500
8	908 978	peaty iron-pan	510
9	906 974	peaty gley	520
10	874 975	peaty gley	390
11	898 997	peaty gley	480
12	898 001	iron-pan	500
13	898 003	peaty gley	490
14	896 003	peaty gley	460
15	903 002	deep peat	515
16	902 023	peaty gley	525
17	901 023	iron-pan, stony sub-soil	510
18	885 020	surface water gley	475
20	853 994	peaty gley	460